Scientific Edition

JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

JUSTIN L. POWERS, EDITOR, WASHINGTON, D. C.

VOLUME XXXIII | *FEBRUARY, 1944* | NUMBER 2 CONSECUTIVE NO. 3

Some Aspects of the Evaluation of Sulfonamide Compounds as Antibacterial Agents*

By George M. Fohlen

Since Gerhardt Domagk's first announcement in 1935 of the value of "Prontosil" on experimental streptococcal infections in mice (1), over two thousand compounds related to sulfanilamide have been prepared by chemists all over the world (2). Most of these have been subjected to more or less crude tests for antibacterial activity. Only a few have been found suitable for use as drugs.

It is the purpose of this paper to point out some of the problems which have arisen in ascertaining whether a compound of the sulfonamide type possesses any therapeutic activity, and what its relative activity is compared to other compounds of the same type. No attempt has been made to consider the clinical evaluation, but it must be realized that the choice of the sulfonamide drug to be used preferentially for any particular infection must be based upon long clinical experience with human infections. Countless numbers of physicians have reported their experiences with the sulfa drugs, and the reviewing of these cases critically and the subsequent recommendations is indeed a formidable task. This has been admirably done in several books and review articles (3–7).

The investigator may have any of several purposes in mind for the evaluation of drugs. He may just want to detect antibacterial activity for which a simple method may suffice, or he may want to determine relative effects quantitatively for the purpose of correlating chemical structure with activity. For this purpose, extreme care must be used.

A very large amount of literature has accumulated concerning the activities of sulfonamide drugs against a variety of bacteria both *in vitro* and *in vivo*. Many of the data are conflicting and require critical examination because the variety of techniques used by the various investigators prevents direct comparison of the results obtained. For instance, some workers have reported that sulfanilamide in concentrations from 10 to 20 mg. % has no effect, others than it is bacteriostatic and still others that it is bactericidal for streptococcus in *in vitro* tests. Obviously, fundamental studies of the variables encountered in *in vitro* experiments had to be undertaken. Some of the major factors which influence the results of *in vitro* tests are the strain, age and virulence of the bacterial culture used, the size of the inoculum, the composition of the nutrient medium, the duration of the experiments,

* Received Sept. 13, 1943, from the University of California, College of Pharmacy, The Medical Center, San Francisco 22, Calif.

This is the fifth in a series of review articles.

the temperature of incubation and the concentration of the drug employed. The effect of some of these variables will now be discussed in greater detail.

The temperature at which incubation is conducted is very important in the *in vitro* testing of the sulfonamide compounds. In 1938, White and Parker (8) found that sulfanilamide was a much more effective bacteriostatic and bactericidal agent against hemolytic streptococcus, both in whole blood and in peptone-glucose-broth media, when the tests were conducted at temperatures from 39° to 40° instead of 37° C. This increase in activity was shown not to be due to a poorer growth response of the controls at these higher temperatures. These results have been confirmed and extended by Weld and Mitchell (9) and White (10). White found that one hundred times as much sulfanilamide was needed to sterilize a culture when incubated at 37° than at 39° C. In view of these results the temperature of incubation should be rigidly controlled if any comparative results are to be obtained. Many investigators neglect to state their incubation temperatures altogether. Even in the same incubators the lower shelf may be 2° or 3° higher than the upper ones and may seriously affect the conclusions from otherwise carefully executed experiments. However, this effect of the temperature on activity may be beneficial in cases of human infections where the body temperature is a few degrees above the normal with fever, or, in cases of combined drug and heat therapy.

The composition of the culture medium used in carrying out the *in vitro* studies of the sulfonamides is an extremely important factor. In 1938, Lockwood (11) found that the addition of small amounts of peptones prepared by the enzymatic digestion of casein and meats to human serum cultures definitely decreased the bacteriostatic effect of sulfanilamide upon virulent hemolytic streptococcus. This peptone antagonism to sulfanilamide has been confirmed and extended by various investigators to other sulfonamide drugs and to other bacteria, and may be accepted as a general phenomenon (12–15). McLeod (13), by using a medium made from pure chemicals and free from inhibitors, has tested many substances for their inhibitor effect. Many substances commonly used in bacteriological media such as yeast, bran and some glandular extracts were found to contain much inhibitor. The amount of inhibitor present in the various materials depends to a large extent upon how they were prepared. Consequently, it may be expected that investigators using different sources or batches of these ingredients will differ as to the concentration of the sulfonamide necessary to achieve bacteriostasis.

Weld and Mitchell (9) suggest that the organisms multiply more slowly in peptone-free serum and, hence, the sulfanilamide has fewer organisms on which to act and has a correspondingly greater effect. They found that a concentration of 100 mg. % of sulfanilamide had only a slight bacteriostatic effect on a streptococcus in rabbit serum whether peptone was present or not, when relatively large inocula (3000 organisms) were used. If the number of bacteria inoculated was small (400), the drug was bactericidal for the organisms in peptone-free serum, and merely bacteriostatic in the peptone containing medium. They conclude from these observations that the better the medium is for the growth of the organisms, the less will be the effect of the sulfanilamide.

Long, *et al.* (14), confirming the experimental data of Lockwood, agree with the general statement of Weld and Mitchell that sulfanilamide becomes less effective with an increase in the nutritive value of the medium for a given organism, but they do not accept their interpretation. They found that 10 mg. % of sulfanilamide was actively bacteriostatic against Group G hemolytic streptococcus in Neopeptone-beef infusion-dextrose broth, while 500 mg. % had scarcely any effect when peptone-dextrose medium was employed, although the organism had essentially the same reproduction rate in the two media.

Fleming (16) observed in 1940 that the use of large inocula of living bacteria, or the addition of a large number of dead cells along with a small number of living ones, results in an interference with the sulfonamide bacteriostasis resembling that of the action of peptones. In this connection, Lockwood suggested that the death and/or lysis of some of the bacteria provide the surviving cells with some split products of proteins similar to the ones found in peptone. Furthermore, Stamp (17), by extracting cultures of hemolytic streptococcus with alkali and concentrating, found that he had an extremely active inhibitor of sulfonamide bacteriostasis. D. D. Woods (18), finding a sulfonamide inhibitory substance in extracts of yeast, concluded that it might be closely related structurally to sulfanilamide. He proceeded to test *p*-aminobenzoic acid for antisulfonamide effects and found that this simple compound possessed such property to a large degree. Woods postulated that *p*-aminobenzoic acid (subsequently referred to as PAB) is an

essential metabolite for certain microörganisms and that sulfonamides act by virtue of their competition with PAB for some enzyme system. This theory has been conclusively proved and widely extended by many investigators (19–23).

This theory of PAB-sulfonamide competition can be used to clarify many of the data existing in the literature for which no satisfactory explanation has been offered. For example, the data of Weld and Mitchell (and also that of Fleming), cited above, can be accounted for by the fact that some PAB was introduced with the organisms, subsequently released by the lysis of the dead organisms, enough, in the cases where the large inocula were used, to overcome the effect of the sulfanilamide. Fox and Rose (24) have shown that 1 part of PAB will overcome the effect of approximately 5000 parts of sulfanilamide at *p*H 7, so that only small amounts of PAB are needed to produce results such as those observed. The data of Lockwood and those of Long, cited above, can be explained by the presence of more PAB in the peptone-containing media than in the other media. It has often been shown that PAB is the principal sulfonamide inhibitor (25) present in these media, although others have been reported (26, 28).

Differences in the activities of the sulfonamide drugs on various organisms, and also on different strains of the same organism, can be attributed to the quantitative differences in the ability of the organism to synthesize PAB. Many strains of bacteria will grow in chemically defined media to which PAB is not added. Those strains which have acquired a resistance to the sulfonamides by continued exposure to them are able to synthesize large amounts of PAB. This was shown to be so recently by Landy and his colleagues. In 1942, Landy and Dicken (29) published a method for the determination of very small amounts of PAB, based upon the growth of the organism, *Acetobacter suboxydans*, which needs PAB for growth and is unable to synthesize it. The amount of growth in 48 hr. at 38° C., as measured photoelectrically, can be correlated with the amount of PAB which has been introduced into the otherwise PAB-free, chemically defined medium. Using this method, they have determined the amount of PAB in various materials and also the sulfonamide inhibitor activities of some compounds related to PAB.

In another study, Landy, Larkum and Oswald (30) have measured the amount of PAB synthesized by 35 different organisms in PAB-free media. All organisms which grew on these media elaborated PAB in amounts readily measured by their technique.

Using the same technique, Landy, Larkum, Oswald and Streightoff (31) have studied the amount of PAB synthesized by sulfonamide-susceptible and -resistant *Staphylococcus aureus*. It was found that the susceptible strains had synthesized 0.049 mg. of PAB per cc. of culture medium, whereas the resistant strain had synthesized 3.59 mg., although the growth of both strains in the PAB-free medium was the same. This increased PAB synthesis by the resistant organisms is sufficient to account for their resistance to the sulfonamide drugs. Moreover, these resistant strains of *S. aureus* continued to produce the excessive amounts of PAB for several generations after exposure to the sulfonamide; that is to say, the sulfonamide-fastness is more or less permanent.

This PAB synthesis by bacteria has a fundamental significance in the evaluation of the effectiveness of sulfonamide drugs, as may readily be imagined. The organisms, in the presence of the sulfonamide, gradually develop a resistance; that is to say, PAB synthesis gradually increases. Kirby and Rantz (32) find that the degree of resistance developed is proportional to the bacteriostatic potency of the drug. Consequently, if one carries out comparative tests for too long periods, the organisms may have sufficient time to develop a resistance to the drugs, more so toward the more active ones so that they may show up rather less effective than they really are. The gradual adaptation of the organisms to the sulfonamide environment has been reported recently by Cooper and Keller (33) who have studied the effects of media, time and size of inocula on the bacteriostatic and bactericidal concentrations of sulfathiazole *in vitro*. They found that certain concentrations of the drug were bacteriostatic for as long as 7 days (0.1 mg. % of sodium sulfathiazole in nutrient broth against *Shigella paradysenteriae* Flexner), after which time growth occurred in the concentration which had been bacteriostatic. For instance, after 14 days 20 mg. %, and after 21 days 60 mg. % were the bacteriostatic concentrations in the same medium. Analogous results were obtained with the other four media used. These results can be quite adequately explained by the gradually increasing production of PAB.

Cooper and Keller (33) also found that the better the nutrient media is, as evidenced by the total number of viable bacteria in the absence of sulfonamide, the less effective is the sulfonamide. This confirms what

had been found by other investigators. These workers also give results on the effect of size of the inoculum upon the concentration of sulfathiazole needed for bacteriostasis after varying periods of incubation up to 3 weeks. On the second day, for example, 1 mg. % was bacteriostatic for all sizes of inocula used, but on the twenty-first day, although 1 mg. % was still bacteriostatic for the inoculum of 33 bacteria per cc., 10 mg. % was required for the inoculum of 330, 40 mg. % for the 3300 inoculum and 100 mg. % for the 33,000 inoculum. These results are consistent with the idea of a gradually increasing PAB synthesis and show the importance of both time and size of inocula in the evaluation of the sulfonamides.

It is not to be assumed that PAB is the only sulfonamide antagonist present in media. Others, such as the amino acid methionine (27, 34) and some purine compounds (35, 36) have been reported to act similarly, but these are much less active than PAB. Conflicting reports have been published concerning the antisulfonamide effects of other compounds. For instance, some workers (37) have regarded nitotinic acid, ascorbic acid and thiamine as sulfonamide antagonists, while other workers have denied this (38). At first sight it would appear that pure, chemically defined media would be ideal to study sulfonamide effects, but it must be emphasized that unless optimal growth of the organism occurs, any substance which stimulates growth might apparently be construed as an antisulfonamide substance (39, 40). The substances such as nicotinic acid, ascorbic acid and thiamine are presumably such nonspecific growth stimulants. Another such compound is biotin. Lampen and Peterson (41) found that biotin is essential to the optimal growth of *Clostridium acetobutylicum* as well as PAB. (This organism needs but cannot synthesize PAB and has been used by McIlwain (42) for PAB assays similar to those of Landy, *et al.*) Rubbo and Gillespie (43), on the other hand, had found that PAB alone was sufficient, and Lampen and Peterson suggest that biotin undoubtedly was introduced into Rubbo and Gillespie's medium with some natural constituent such as glucose.

The action of methionine does not appear to be the result of its acting as a growth stimulant. The addition of xanthine or guanine increases the inhibitory action of the methionine (35). However, in the absence of methionine, these compounds increase the action of the sulfonamide. Hypoxanthine and adenine increase the bacteriostatic effects of the sulfonamides whether methionine is present or not. In the absence of sulfonamide, all of these purines show no stimulation of growth of the test organism. Landy and Streightoff (44) also found that purines seem to act as growth accessory substances for *Acetobacter suboxydans*, the organism used in their PAB assay, but that they were not essential metabolites. For instance, they had no effect when PAB was present in excess of 0.03 mg./cc.; nor were they effective when there was no PAB at all. However, when PAB was present in amounts less than 0.03 mg./cc., the addition of the purines stimulated the growth of the organisms, so that in the assay, the effect would be interpreted as being due to more PAB than is actually present. They got around this by adding some adenine, guanine and xanthine to their basal medium.

Other sulfonamide antagonists of importance, although not occurring naturally, are the local anesthetics derived from PAB by esterification, such as procaine, "Tutocain," "Larocaine" and others. Woods (18), in 1940, showed that procaine was almost as strongly antisulfanilamide as PAB. Keltch, Baker, Krahl and Clowes (45) have shown the antisulfathiazole and antisulfapyridine effect *in vitro* of seven local anesthetics derived from PAB. None of nine local anesthetics not derived from PAB displayed any such antagonism. This same antogonism has been demonstrated *in vivo* and has great clinical importance. If local anesthetics are to be used on patients receiving sulfonamide drugs, those not derived from PAB should be employed.

One of the more recent developments in the theory of the mode of action of sulfonamides is that only the ionized portion of the compounds exerts the antibacterial action. Fox and Rose (24) have concluded this to be so. Furthermore, they found that the minimal amounts of ionized drug (sulfanilamide, sulfapyridine, sulfathiazole and sulfaguanidine were used) required to achieve bacteriostasis under similar test conditions were approximately the same. Great difference noted in the amounts of sulfonamide needed to cause bacteriostasis at different *p*H's, although growth in the controls was the same, can be attributed to the corresponding differences in the amounts of ionized drugs. The same idea has been expressed by other investigators. Schmelkes (46, 47), *et al.*, have shown that the antibacterial efficiency of sulfonamides, as measured by their ability to overcome PAB, is dependent upon the *p*H of the medium. At a high *p*H, where a proportionately larger amount of the dis-

solved sulfonamide exists as an anion, it will compete on a more favorable basis with the *p*-aminobenzoate ion than at lower *p*H's where the dissociation of the sulfonamide occurs to a much less extent. As the *p*H is gradually increased above 7, sulfanilamide ($K_a = 2.2 \times 10^{-11}$) approaches the effectiveness of sulfathiazole ($K_a = 6.2 \times 10^{-8}$). This clearly shows that the *p*H of the medium used to carry out comparative tests on sulfonamide compounds must be defined and buffered to prevent shifting *p*H's of the medium as the test proceeds. Also, clinically, it would appear advantageous to maintain the *p*H of infected loci at the highest physiologically possible value.

The effects of a number of factors upon the results of the antibacterial action of the sulfonamides *in vitro* have been discussed. They show that if comparisons of the various compounds are to mean much, rigid precautions must be taken. Summarizing these precautions:

1. The temperature of incubation must be carefully controlled, regulated to within a tenth of a degree range.
2. The medium used must be as nutritive as possible for the particular organism, so that optimal growth occurs in the controls, but must not contain any sulfonamide inhibitors.
3. The period of incubation has to be such that the organism does not have time to develop abnormally increased synthetic powers for PAB. Three or four days seem to be the maximum. The organism must not be sulfonamide-resistant to start with.
4. Comparative tests must be made with the same organism and the same medium.
5. The size of the inocula must be the same in each culture and must not be too large.
6. The medium must be buffered to maintain the same *p*H throughout the period of incubation.

If the problems involved in the *in vitro* testing of the sulfonamides seem to be complex, then the *in vivo* evaluation is indeed more so, because the animal factor multiples the difficulties. The mode and rate of absorption, distribution, detoxification and excretion, the method of administration of the drug and the subsequent treatment of the animals are all further variables to contend with as well as most of those considered in the *in vitro* tests.

Many species of animals have been tried for test purposes—mice, rats, dogs, chickens, guinea pigs, turtles, sharks, gold-fish, etc. Different animals behave in different manners. However, most of the *in vivo* work has been done with Swiss white mice. These are cheap to use, easily handled in large numbers, are obtainable in pure strains and are susceptible to many of the diseases of man.

In the case of *in vivo* tests, too, as with the *in vitro* tests, it is not too difficult to detect antibacterial activity. Simple tests showing the prolongation of the lives of treated, infected mice over controls of untreated infected mice suffice to show this. For instance, Domagk (1), in the first published paper of *in vivo* tests of "Prontosil," infected 25 white mice with 0.3 cc. of a 1:1000 dilution of a 24-egg-broth culture of hemolytic streptococcus obtained from a patient with a fatal septicemia. Of these, 14 were retained as controls and the remaining 11 were given varying amounts of "Prontosil" (from 0.2 cc. of a 0.1% solution to 1.0 cc. of a 1% suspension) by mouth, $1\frac{1}{2}$ hr. after the infection. The controls all died within 72 hr., whereas all the treated animals survived longer than a week and were apparently healthy.

Data on the effectiveness of the sulfonamide compounds have been obtained by the administration of doses according to schedules which have varied with nearly every investigator. Some have given but one dose of the drug either before or for varying periods after the infection, some have given the drug once a day, and yet still others, several times a day. Indeed, great variations of the concentration of the drug in the blood must have resulted from these various dosage schedules. In some cases there has been a high drug level for a short time, followed by a time during which there was almost none, and in other cases there has been an almost constant level. Furthermore, a lack of agreement as to a suitable therapeutic response as an end point, and of a standard of comparison, as well as complete neglect or ignorance of the accuracy of comparisons, has invalidated the quantitative conclusions from many experiments. Only a qualitative significance can be attached to the majority of the work.

In 1939, Litchfield, White and Marshall published a report entitled "The Experimental Basis for a Method for the Quantitative Evaluation of the Effectiveness of Chemotherapeutic Agents Against Streptococcus Infections in Mice" (48). In this report the authors describe the elaborate experiments which they have performed to study the influences of many factors in order to establish a more nearly quantitative method of measuring and comparing the sulfonamide compounds. Since this report is a fundamental and a definitive one, it will be discussed in some detail.

The test animals used were Swiss white mice of uniform stock, both males and females, within the weight range of 15 to 20 Gm. Assuming, reasonably, and from evidence obtained by previous workers, that a drug of this type is most effective therapeutically when its concentration in the blood and tissues is maintained at a more or less constant level throughout the period of therapy, those workers achieved this condition quite satisfactorily by incorporating the drug into the food (Purina Dog Chow) fed to the mice. By putting different amounts of drug in the food, the drug intake can be varied within an adequate range. It was found that, except for the first day, the mice ate 6 to 8 times, evenly spaced, during each 24-hr. period. The amount of food varied cyclicly; however, there was at all times some drug present in the intestinal tract. When mixed with the food, sulfanilamide was found to be more slowly and evenly absorbed than from an acacia solution containing the same concentration of drug. From some of their data on drug intakes, the authors were able to calculate the variations, not only from mouse to mouse, but also from day to day among individual mice. The magnitude of these variations indicated that all the external factors of the environment should be controlled as closely as possible and that all mice should be treated individually in separate cages. Any other method of handling which involves the grouping of the mice would prevent the direct correlation of life or death with drug intake. The food cups were built so as to prevent any food wastage and the mice were allowed to feed at will. From the weight of the food cups at certain intervals, the amount of drug consumed can be determined and by means of a previously determined conversion factor, the concentration of the drug in the blood can be calculated.

In this study, Litchfield, White and Marshall produced a standard infection by Strain 203, Group A (Lancefield's classification) of β-hemolytic streptococcus. This is a highly virulent organism, a single coccus or chain being a lethal dose for a mouse. The mice were infected with either 200 or 20,000 L. D. or the organisms. The mean survival time of 178 control mice for the 200 L. D. infection was 22.3 ± 2.5 hr.

The onset of treatment is one factor which has varied considerably among many investigators. Domagk (1) treated his mice with "Prontosil" $1^1/_2$ hr. after infection; other workers have used different intervals. In order to determine the effect of the onset, Litchfield, White and Marshall tried three different plans of treatment. In one, the drug diet was prefed 1 to 3 days prior to infection. In another, the mice received one-eighth of the expected daily drug intake at the time of infection, and then placed on the drug diet. The third method of treatment was similar except that the treatment was started 4 hr. after the infection. The mice received the drug (sulfanilamide was used) for 3 days. No significant difference was found between the prefeeding and the immediate types of treatment, but a significant decrease in the number of surviving animals was found in the cases of delayed treatment.

The relationship between the blood concentration level and the duration of such a concentration on the number of mice surviving the 200 L. D. infection was studied. It was found that a well-graded response was obtained with a 3-day treatment after infection.

All mice surviving infections in all the experiments carried out were observed for a period of 30 or more days. A 30-day observation period was found to be more than adequate. The majority of the mice not surviving died before the 7th day.

It is interesting to note that in these studies the food intake seemed to vary independently of the weight of the mouse, so that the drug intakes per mouse were used instead of the weight of drug per Gm. (or Kg.) of body weight.

In all of these experiments, large groups of mice were used, the standard errors were evaluated and chi-square tests for the significancy of differences were applied. From these foundations, the authors have developed a method for evaluating quantitatively not only the therapeutic effectiveness of a drug under different conditions, but also of different drugs under the same conditions. In essence, the method is as follows:

"In individual cages the drug-diet intake can be determined. Drug diets are selected so that one may expect survival percentages greater and less than 50. These diets are prefed for one or more days prior to and for the desired period after the infection. Irrespective of the per cent of drug in any diet, the average daily drug intakes (per mouse) can be arranged in groups and correlated with the per cent survival. The dosage-survival curve is now computed and the Median Survival Dose ($S.D._{50}$) is obtained from the curve. This can now be converted to Median Survival Blood Concentration ($S.B.C._{50}$) by a factor which relates blood concentration to the daily drug intake. By using a standard, one obtains

comparative results for the $S.B.C._{80}$'s which may be nearly absolute, although the $S.B.C._{80}$'s themselves are variable."

A limitation of the drug diet method is that some drugs may render the diet distasteful so that the mice would not feed upon it freely. Also infections with certain organisms may render the mice so sick that they may be unable to feed on the drug diet as normally. There is a disadvantage in the large number of individual mouse cages required for any extensive program of testing and also the numerous weighings and calculations required, but the reliable results so obtained makes the effort worth the while.

Among the advantages of the drug-diet method over some of the ones used by other workers are that a more or less constant blood level of the drug is maintained. If this were done by more frequent dosing either parenterally or by tubing, the mice would be so upset that extremely variable results would be obtained. With this method the mice are not handled after being infected. Since the blood levels of the drugs are used as the basis for comparisons, the effects of absorption excretion and distribution of the drugs do not serve as further variables, and an indication of the magnitude of these factors may be obtained from the value of the conversion factor used for calculating the blood concentration from the amount of the drug ingested. In this way it is possible to ascertain whether a compound is inherently inactive, or only apparently so because of poor absorption or too rapid elimination. Also it is possible to ascertain whether the compound might be inactive because of rapid conjugation in the body to form inactive products, because blood level determinations in the control animals (in order to determine the conversion factor) will indicate such conjugation. Toxicity to the host will be apparent in the controls also. A 100% survival (30 mice) was obtained by a daily dose of 7 mg. of sulfanilamide.

Using this method, these workers have obtained the activity ratios of some of the common sulfonamide compounds *versus* streptococcus (49) and pneumococcus (50) infections. These are given below their standard errors, using sulfanilamide as the standard of comparison:

Drug	Activity Ratios	
	Strepto-coccus	*Pneumo-coccus*
Sulfanilamide.....	1	1
Sulfapyridine.....	1.09 ± 0.13	2.32 ± 0.43
Diaminodiphenyl-sulfone.....	2.92 ± 0.36	15.92 ± 1.83
Sulfathiazole.....	0.80 ± 0.18	2.82 ± 0.14
Sulfaguanidine.....	0.66 ± 0.16	2.81 ± 0.75

Domagk (1), in his original paper, stressed the fact that "Prontosil" was effective against streptococcus only in the living organism and was without bacteriostatic or bactericidal effect *in vitro*. It is indeed fortunate that *in vivo* tests were done although the *in vitro* tests showed no effect, since this inactivity *in vitro* is in contrast to the actions of chemicals formerly tried. It was soon shown, however, by Trèfouël, Trèfouël, Nitti and Bovet (51) that "Prontosil" was changed in the body to sulfanilamide. Sulfanilamide was subsequently shown to be active both *in vivo* and *in vitro*. In order to simplify the evaluation of the multitudinous compounds synthesized, it is desirable that some relationship between the *in vitro* and the *in vivo* activities be established. White, Bratton, Litchfield and Marshall (52) did undertake a careful study of such a relationship in a semiquantitative sense. One hundred and twenty-six compounds were studied, consisting mainly of sulfanilamide derivatives with a few sulfones, sulfoxides and sulfides. The infections against which the compounds were tested were those of β-hemolytic streptococcus, Type I pneumococcus and a strain of *Streptococcus viridans*. The *in vivo* tests were done on mice using a drug-diet method; units of 10 mice each were used for each compound. A 0.5% drug diet was prefed for 3 days and continued for 3 days after infection. The *in vitro* rests were performed in quadruplicate at least, 10 mg. % being the concentration of drug used. Conditions employed were such that sulfanilamide was bactericidal in amounts less than 10 mg. %. Actually, the minimal bactericidal concentration of sulfanilamide under these conditions was 2.5 ± 0.5 mg. % against the 3 bacterial species used. The conclusions drawn by these workers from these experiments were:

"1. No compound is active *in vivo* if it is inactive *in vitro*, unless it is decomposed in the animal organism to a compound which would be active *in vitro*.

"2. Several compounds are very active *in vitro* but inactive *in vivo*.

"3. A certain amount of specificity for the three species of bacteria appears to exist *in vitro*."

These observations are in accord with the generally accepted idea that the primary action of these types of drugs is upon the bacterial organism. And the work offers a basis for a rapid method for the examination of a large number of compounds for selection for a more detailed study *in vivo*.

As in the case of *in vitro* studies, PAB has

been found to antagonize the action of sulfanilamide *versus* hemolytic streptococcus in mice (53). However, *in vivo* it took many times the amount of PAB necessary to inhibit a like amount of sulfanilamide *in vitro*. McIlwain (42) has recently shown that PAB may be partially inactivated in the body by acetylation. The variation in the concentration and metabolism of the sulfonamide inhibitors in hosts of different species are factors to be considered in transferring results from animal experiments to human practice.

Too frequently a new compound is compared with others only against one or two types of organisms, and if it is inactive in these tests, the compound is quite apt to be forgotten. It is quite possible that some such forgotten compound may be just the thing to treat some other infection against which the known sulfonamides are not particularly effective. However, in this connection a precaution is to be noted which has often been neglected. This is the variation in the effect of organisms in the host infected experimentally from the naturally acquired infection in man. For instance, some sulfonamides have been found to be effective against experimental typhoid infection in mice, but in man they have little or no effect. This is because the typhoid infection in mice leads to a septicemia which is the cause of death and which can be effectively treated by these compounds, whereas in man the main feature of the disease is an infection of the intestinal wall which persists for several weeks and against which these drugs are powerless (54).

The foundations for the evaluation of the sulfonamide types of compounds both *in vivo* and *in vitro* have been laid by the many workers in this field. Some of the work that has been done in evaluating drugs of this type has been very good, and other work has been a waste of time and materials because of neglect of some factor. Perhaps, many factors still remain hidden. It is to be hoped that in future work these factors will be heeded and aid the progress of chemotherapy to the still unconquered diseases.

REFERENCES

(1) Domagk, G., *Deut. med. Wochschr.*, 61 (1935), 250.
(2) Northey, E. H., *Chem. Revs.*, 27 (1940), 85.
(3) Long, P. H., and Bliss, E. A., "The Clinical and Experimental Use of Sulfanilamide, Sulfapyridine and Allied Compounds," Macmillan Co., New York, 1939.
(4) Keefer, C. S., "Advances in Internal Medicine," Interscience Publishers, Inc., New York, 1942, p. 102.
(5) Goodman, L., and Gilman, A., "The Pharmacological Basis of Therapeutics," The Macmillan Co., New York, 1941.
(6) Schnitker, M. A., "The Sulfonamide Compounds in the Treatment of Infections," Oxford Univ. Press, New York, 1942.
(7) Spink, W. W., "Sulfanilamide and Related Compounds in General Practice," Second edition, The Yearbook Publishers, Chicago, 1942.
(8) White, H. S., and Parker, J. M., *J. Bact.*, 36 (1938), 481.
(9) Weld, J. T., and Mitchell, L. C., *Ibid.*, 38 (1939), 335.
(10) White, H. J., *Ibid.*, 38 (1939), 549; also *Proc. Soc. Exptl. Biol. Med.*, 43 (1940), 214.
(11) Lockwood, J. S., *J. Immunol.*, 35 (1938), 155.
(12) Lockwood, J. S., and Lynch, H. M., *J. Am. Med. Assoc.*, 114 (1940), 935.
(13) McLeod, C., *J. Exptl. Med.*, 72 (1940), 217.
(14) Long, P. H., in reference 3 (above).
(15) Gay, F. P., Clark, A. R., Street, J., and Miles, D., *J. Exptl. Med.*, 69 (1939), 607.
(16) Fleming, A., *J. Path. Bact.*, 50 (1940), 69.
(17) Stamp, T. C., *Lancet*, 2 (1939), 10.
(18) Woods, D. D., *Brit. J. Exptl. Path.*, 21 (1940), 74.
(19) Fildes, P., *Lancet*, 1 (1940), 956.
(20) McIlwain, H., *Ibid.*, 1 (1942), 412.
(21) Wyss, O., *Proc. Soc. Exptl. Biol. Med.*, 48 (1941), 122.
(22) Green, H. N., and Bielschowsky, F., *Brit. J. Exptl. Path.*, 23 (1942), 1, 13.
(23) Fox, C. L., Jr., and Rose, H. M., *Proc. Soc. Exptl. Biol. Med.*, 47 (1941), 159.
(24) Fox, C. L., Jr., and Rose, H. M., *Ibid.*, 50 (1942), 142.
(25) Blanchard, K. C., *J. Biol. Chem.*, 140 (1941), 919.
(26) Loomis, T. A., Hubbard, R. S., and Neter, E., *Proc. Soc. Exptl. Biol. Med.*, 47 (1941), 159.
(27) Kohn, H. I., and Harris, J. S., *J. Pharmacol.*, 73 (1941), 383.
(28) Snell, E. E., and Mitchell, H. K., *Arch. Biochem.*, 1 (1942), 93.
(29) Landy, M., and Dicken, D. M., *J. Biol. Chem.*, 146 (1942), 109.
(30) Landy, M., Larkum, N. W., and Oswald, E. J., *Proc. Soc. Exptl. Biol. Med.*, 52 (1942), 338.
(31) Landy, M., Larkum, N. W., Oswald, E. J., and Streightoff, F., *Science*, 97 (1943), 265.
(32) Kirby, W. M. M., and Rantz, L. A., *J. Exptl. Med.*, 77 (1943), 29.
(33) Cooper, M. L., and Keller, H. M., *Proc. Soc. Exptl. Biol. Med.*, 50 (1943), 148.
(34) Bliss, E. A., and Long, P. H., *Bull. Johns Hopkins Hosp.*, 69 (1941), 14.
(35) Harris, J. S., and Kohn, H. I., *J. Biol. Chem.*, 141 (1941), 989.
(36) Martin, G. S., and Fischer, C. V., *Ibid.*, 144 (1942), 289.
(37) Hill, J. H., and Mann, E. F., *J. Urol.*, 47 (1942), 522.
(38) Raiziss, G. W., and Moetsch, J. C., THIS JOURNAL, 31 (1942), 198.
(39) Green, H. N., *Brit. J. Exptl. Path.*, 21 (1940), 74.
(40) MacLeod, C. M., "Advances in Internal Medicine," Volume I, Interscience Publishers, Inc., New York, 1942, p. 83.
(41) Lampen, J. O., and Peterson, W. H., *J. Am. Chem. Soc.*, 63 (1941), 2283.
(42) McIlwain, H., *Brit. J. Exptl. Med.*, 23 (1943), 265.
(43) Rubbo, S. D., and Gillespie, J. M., *Nature*,

146 (1940), 838.
(44) Landy, M., and Streightoff, F., *Proc. Soc. Exptl. Biol. Med.*, 57 (1943), 127.
(45) Keltch, A. K., Baker, L. A., Krahl, M. E., and Clowes, G. H., *Ibid.*, 47 (1941), 533.
(46) Schmelkes, F. C., and Wyss, O., *J. Bact.*, 43 (1942), 71.
(47) Schmelkes, F. C., Wyss, O., Marks, H. C., Ludwig, B. J., and Strandskov, F. B., *Proc. Soc. Exptl., Biol. Med.*, 50 (1942), 145.
(48) Litchfield, J. T., Jr., White, H. J., and Marshall, E. K., Jr., *J. Pharmacol.*, 67 (1939), 437.
(49) Marshall, E. K., Jr., Litchfield, J. T., Jr., and White, R. J., *Ibid.*, 69 (1940), 89.
(50) Litchfield, J. T., Jr., White, H. J., and Marshall, E. K., Jr., *Ibid.*, 69 (1940), 166.
(51) Tréfouël, J., Tréfouël, Mme. J., Nitti, F., and Bovet, D., *Compt. rend. soc. biol.*, 120 (1936), 756.
(52) White, H. S., Bratton, A. C., Litchfield, J. T., Jr., and Marshall, E. K., Jr., *J. Pharmacol.*, 72 (1941), 112.
(53) Selbie, F. R., *Brit. J. Exptl. Path.*, 21 (1940), 90.
(54) O'Meara, R. A. Q., *Irish J. Med. Sci.*, 193 (6) (1942), 1.

Oil of Peppermint. Storage Factors and Use of Antioxidants as Preservatives*

By L. H. Baldinger,† N. K. Ellis‡ and K. I. Fawcett‡

Improper storage of peppermint oil, the chief factor in the deterioration of this product, causes a darkening of the oil, as well as the production of an off-odor and resinous appearance. This paper includes a report of an investigation of storage conditions, experiments on the use of antioxidants for the preservation of the oil and a study of the combined effects of antioxidants and proper storage conditions.

PRELIMINARY STORAGE EXPERIMENTS

In order to make a preliminary study of the effects of a few storage conditions encountered in the commercial production of mint oil, two samples of fresh mint oil were divided into portions early in the fall of 1937. Half of the portions were stored in an attic, which was subjected to temperature extremes similar to those encountered in storage by mint growers; the remaining portions were stored in a cool cellar. The containers for the oil samples included clear-glass bottles with cork stoppers, clear-glass bottles with corncobs as stoppers, tin cans with cork stoppers, and sealed tin cans. At various intervals during the following year of 1938—February, June, October—the storage samples were examined chemically and physically to determine the changes which had taken place.

The following points were observed from an examination of the data for these oils. The optical rotation increased slightly in those samples which were not protected from air, that is, in containers stoppered with corncobs or poorly stoppered with cork. No great differences were observed for this constant between cool-storage and attic-storage conditions. Ester and menthol values showed great apparent increases under poor storage conditions particularly in tin cans with cork stoppers and in glass bottles with corncob stoppers. It should be emphasized that the increase in ester and menthol contents is only apparent. Inasmuch as the calculation of the content of each of these constituents is based upon the amount of potassium hydroxide used in the assay, the formation of any compounds which will also react with this base will cause an erroneously high result in the estimation of the active constituent. It is believed that in the oxidative changes, which take place under poor storage, resins are formed which cause a discoloration of the oil, and which have been shown to react with potassium hydroxide. The apparent increase in menthol and ester content serves to indicate, therefore, relative amounts of resins and to classify roughly storage conditions. Menthone contents increased slightly, but not in proportion to the menthol and ester contents. All samples showing increased physical or chemical constants had darkened upon standing and were definitely substandard at the end of the storage period. Storage samples in glass bottles, tightly stoppered with cork, or in sealed cans, either in cool storage or in an attic, showed minimum changes.

PRELIMINARY EXPERIMENTS WITH ANTIOXIDANTS

In order to investigate the effect of antioxidants in the storage of peppermint oil, preliminary work involved the aeration of several oil samples to which different antioxidants had been added, and subsequent analysis of the samples. Another group of protected samples was stored in sunlight for 6 months and subsequently analyzed.

Admittedly, the use of antioxidants as a substitute for proper storage conditions should be discouraged, but the effects of very small quantities of these substances suggests the possibilities of their use when proper storage conditions are not available.

The use of antioxidants in fixed oils is quite common (1) and it would seem that equally good effects could result from their use in essential oils. In choosing antioxidants for use by producers of peppermint oil, cheapness and accessibility are two im-

* This manuscript was prepared in connection with Peppermint Oil investigations conducted by the Purdue University Agricultural Experiment Station. For a detailed report of these investigations see Purdue University Agricultural Experiment Station Bulletin No. 461, "A Study of Some of the Factors Affecting the Yield and Market Quality of Peppermint Oil," Purdue University, Lafayette, Ind.

† Department of Chemistry, University of Notre Dame, Ind.

‡ Department of Horticulture, Purdue University, Lafayette, Ind.

portant factors to be considered; for that reason, only a few of the more commonly used and cheaper antioxidants were tried, α-naphthol, hydroquinone, catechol, pyrogallol and tartaric acid. The latter was included because of the common practice of mint oil producers in using this material for decolorizing purposes. The aeration procedure was relatively simple. Compressed air, about two bubbles per second, was forced through 60-ml. portions of the oil. Inasmuch as the bubbing jets were of different size, the aeration was not uniform in all samples. The data collected for these samples indicate that α-naphthol, hydroquinone, catechol and pyrogallol, in 0.1% concentration, are of value in inhibiting changes in this oil, changes which are indicated by apparent higher menthol and ester contents. Pyrogallol and α-naphthol are valueless because of the dark color imparted to the oil; tartaric acid does not serve to inhibit oxidation.

in the oil samples with respect to this component parallel the other changes.

ANALYTICAL PROCEDURES

The analyses of the samples included in this work were performed according to the methods of the Pharmacopœia of the United States of America, Eleventh Revision, as listed under the monograph for Oil of Peppermint. One modification of the method is worthy of note. In the washing of the acetylated oil, hot water was used to decompose the excess anhydride and to remove acetic acid before subsequent washing with sodium carbonate test solution. The authors believe that this procedure has eliminated possible errors in the saponification of the acetylated sample due to undecomposed acetic anhydride and has made for more consistent results in checking analyses.

TABLE I.—PHYSICAL AND CHEMICAL DATA FOR SAMPLES OF PEPPERMINT OIL STORED IN A COOL CELLAR IN DIFFERENT CONTAINERS AND PROTECTED WITH ANTIOXIDANTS[a]

Type of Container and Treatment	Optical Rotation		Refractive Index		Esters, %		Total Menthol, %	
	June, 1940	Dec., 1940	June, 1940	Dec., 1940	June, 1940	Dec., 1940	June, 1940	Dec., 1940
Glass bottle, cork stopper	$-21.9^{25°}$	$-21.4^{25°}$	$1.4620^{17°}$	$1.4610^{20°}$	7.3	11.7	45.8	49.6
Glass bottle, corncob stopper	$-21.8^{25°}$	$-21.5^{25°}$	$1.4616^{17°}$	$1.4614^{20°}$	7.7	11.9	45.8	50.7
Tin can, cork stopper	$-22.7^{25°}$	$-22.5^{25°}$	$1.4620^{17°}$	$1.4600^{20°}$	5.3	5.1	43.7	44.0
Tin can, sealed	$-23.2^{25°}$	$-23.5^{25°}$	$1.4610^{17°}$	$1.4595^{20°}$	4.0	4.0	42.9	43.1
Glass bottle, cork stopper, 0.1% catechol	$-23.5^{25°}$	$-23.5^{25°}$	$1.4602^{17°}$	$1.4597^{20°}$	4.9	4.2	43.3	42.4
Glass bottle, corncob stopper, 0.1% catechol	$-23.5^{25°}$	$-23.8^{25°}$	$1.4610^{17°}$	$1.4598^{20°}$	4.9	4.9	42.9	43.7
Tin can, cork stopper, 0.1% catechol	$-23.5^{25°}$	$-23.7^{25°}$	$1.4608^{17°}$	$1.4596^{20°}$	4.5	4.7	43.0	43.6
Tin can, sealed, 0.1% catechol	$-23.4^{25°}$	$-23.6^{25°}$	$1.4611^{17°}$	$1.4597^{20°}$	4.2	4.5	43.1	43.2
Glass bottle, cork stopper, 0.1% hydroquinone	$-23.5^{25°}$	$-23.5^{25°}$	$1.4603^{17°}$	$1.4599^{20°}$	4.9	4.9	43.0	42.8
Glass bottle, corncob stopper, 0.1% hydroquinone	$-23.5^{25°}$	$-23.7^{25°}$	$1.4608^{17°}$	$1.4598^{20°}$	4.8	4.9	43.0	43.5
Tin can, cork stopper, 0.1% hydroquinone	$-23.3^{25°}$	$-23.7^{25°}$	$1.4609^{17°}$	$1.4595^{20°}$	4.8	4.5	43.0	43.4
Tin can, sealed, 0.1% hydroquinone	$-23.4^{25°}$	$-23.6^{25°}$	$1.4610^{17°}$	$1.4597^{20°}$	4.4	4.6	43.1	43.4
Tin can, sealed, stored in a refrigerator for entire storage period	$-24.5^{27°}$	$-23.6^{25°}$	$1.4617^{17°}$	$1.4595^{20°}$	4.0	3.8	42.9	42.3

[a] The sample as originally analyzed in October, 1939, showed the following constants: Optical Rotation, −23.4 at 25° C.; Refractive Index, 1.4577 at 24° C.; Esters, 4.0%; Total Menthol, 42.9%. Triplicate rechecks in August, 1940, of the original sample which had been stored in a tightly stoppered clear-glass bottle at room temperatures showed the following percentages of total menthol: 42.9, 42.9; 42.9, 42.9; 42.9, 43.0. One portion of the oil was acetylated with one-year-old acetic anhydride, another with two-months-old anhydride and the third with fresh acetic anhydride. These experiments were performed to determine if the age of the reagent would cause any variation in results.

STORAGE EXPERIMENTS WITH OIL PROTECTED BY ANTIOXIDANTS

Aeration in the laboratory can only approximate the conditions under which some oil samples are stored. The preliminary work on the storage of mint oil under different conditions and in a variety of containers, and the effect of antioxidants on the aging of oils, was followed by supplementary experiments in which a series of samples was placed in storage in October, 1939, to show the combined effect of these factors.

In Tables I and II are tabulated the types of containers and treatment involved, also chemical and physical constants for the oil samples, as determined at various intervals. In this series of samples menthone determinations were omitted. No definite and reliable procedure for determining this component has been included in the Pharmacopœia; furthermore, in the case of previous storage samples on which menthone assays had been made, according to published methods (2), it was shown that changes

DISCUSSION AND SUMMARY

If the per cent of menthol is used as a criterion upon which to base the efficiency of antioxidant action and to compare storage conditions, it has been observed that cool-cellar storage is better than storage in an attic or in locations where extremes of temperature are likely.

In comparing the various types of containers, it is believed that the sealed tin is the most advantageous of the containers which were tested.

Catechol as an antioxidant in 0.1% concentration appears to be more effective than hydroquinone in maintaining the original menthol percentage. Perhaps the chief disadvantage in the use of either of these anti-

TABLE II.—PHYSICAL AND CHEMICAL DATA FOR SAMPLES OF PEPPERMINT OIL STORED IN AN ATTIC IN DIFFERENT CONTAINERS AND PROTECTED WITH ANTIOXIDANTS[a]

Type of Container and Treatment	Optical Rotation		Refractive Index		Esters, %		Total Menthol, %	
	June, 1940	Dec., 1940	June, 1940	Dec., 1940	June, 1940	Dec., 1940	June, 1940	Dec., 1940
Glass bottle, cork stopper	$-21.5^{25°}$	$-20.6^{25°}$	$1.4617^{17°}$	$1.4618^{20°}$	9.9	15.4	48.8	54.3
Glass bottle, corncob stopper	$-21.6^{25°}$	$-20.3^{25°}$	$1.4616^{17°}$	$1.4622^{20°}$	10.9	17.2	48.7	54.5
Tin can, cork stopper	$-21.4^{25°}$	$-21.0^{25°}$	$1.4610^{17°}$	$1.4600^{20°}$	5.3	5.1	43.7	44.0
Tin can, sealed	$-23.4^{25°}$	$-23.7^{25°}$	$1.4610^{17°}$	$1.4599^{20°}$	3.7	4.0	43.3	42.8
Glass bottle, cork stopper, 0.1% catechol	$-23.4^{25°}$	$-23.5^{25°}$	$1.4606^{17°}$	$1.4600^{20°}$	4.5	5.0	43.5	44.1
Glass bottle, corncob stopper, 0.1% catechol	$-23.5^{27°}$	$-23.5^{25°}$	$1.4606^{17°}$	$1.4600^{20°}$	4.6	4.7	43.2	43.5
Tin can, cork stopper, 0.1% catechol	$-23.5^{27°}$	$-23.7^{25°}$	$1.4607^{17°}$	$1.4600^{20°}$	4.8	5.0	43.4	43.4
Tin can, sealed, 0.1% catechol	$-23.4^{27°}$	$-23.6^{25°}$	$1.4604^{17°}$	$1.4598^{20°}$	4.5	4.3	42.9	42.7
Glass bottle, cork stopper, 0.1% hydroquinone	$-23.5^{25°}$	$-23.4^{25°}$	$1.4605^{17°}$	$1.4600^{20°}$	4.9	4.8	43.6	43.3
Glass bottle, corncob stopper, 0.1% hydroquinone	$-23.5^{25°}$	$-23.7^{25°}$	$1.4606^{17°}$	$1.4599^{20°}$	4.8	4.7	43.0	43.3
Tin can, cork stopper, 0.1% hydroquinone	$-23.6^{25°}$	$-23.6^{25°}$	$1.4608^{17°}$	$1.4599^{20°}$	4.6	4.6	43.7	43.6
Tin can, sealed, 0.1% hydroquinone	$-23.4^{27°}$	$-23.6^{25°}$	$1.4606^{17°}$	$1.4598^{20°}$	4.2	4.4	43.4	43.3

[a] See Table I for data concerning the original sample, also for the control sample which was stored in a sealed tin can at refrigerator temperatures.

oxidants is the slight coloration produced, especially in the older oils. This coloration is more noticeable in the case of hydroquinone-protected samples, much less pronounced in the catechol-protected samples and less noticeable for both antioxidants in the samples stored in corked bottles and sealed tins. The slight coloration is not accompanied by a resinous odor which is very noticeable in unprotected oils. The authors are reluctant to recommend the use of antioxidants except to those who are cognizant of the limitations of such agents for the preservation of easily oxidized materials such as peppermint oil.

For the growers and producers, it is recommended, wherever feasible, that sealed containers, completely filled, be used for storing oil and that cool storage conditions be maintained at all times.

REFERENCES

(1) Olcott, H. S., *J. Am. Chem. Soc.*, 56 (1934), 2492; French, R. B., Olcott, H. S., and Mattill, H. A., *Ind. Eng. Chem.*, 27 (1935), 724.
(2) Baldinger, L. H., *Proc. Indiana Acad. Sci.*, 52 (1943), 111.

Synthesis and Chemical Properties of Diasone*

By G. W. Raiziss, L. W. Clemence and M. Freifelder

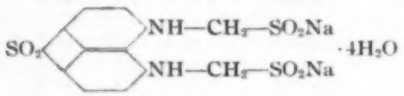
Diasone, the name we gave to disodium formaldehyde sulfoxylate diaminodiphenylsulfone, was prepared by us in December, 1937, first described in a patent application, May 25, 1938, and again in U. S. pat. 2,256,575.[1] Although the early investigation of this product was concerned with its toxicology and therapeutic properties in streptococcic and pneumococcic infections of mice, more recently diasone assumed an important role because of its curative effect in experimental tuberculosis of guinea pigs. Callomon (1) found diasone to be less toxic than similar products and yet as beneficial in its therapeutic effects on tubercular guinea pigs. Feldman, Hinshaw and Moses (2) likewise found diasone to be an effective therapeutic agent in experimental tuberculosis. A further study of its chemistry as well as its biochemistry may, therefore, be of interest.

The product, formed as a result of the reaction between 4,4′-diaminodiphenylsulfone and sodium formaldehyde sulfoxylate, cannot be recrystallized and contains small amounts of other admixtures, in addition to the major product. It is, however, suffi-

* Received Oct. 26, 1943, from the Dermatological Research Laboratories, Philadelphia, Pa., Division of Abbott Laboratories, North Chicago, Ill.

[1] An analogous product was prepared simultaneously and independently by Rosenthal and Bauer, and described in U. S. pat. 2,234,981.

ciently pure and uniform to be used with success in the treatment of infected animals, as well as patients. Diasone is water soluble and remains so indefinitely if kept in glass ampuls sealed under high vacuum. Exposed to the air for several days the powder undergoes some change with the result that a part of it becomes insoluble. Mixed, however, with sodium bicarbonate, it remains unchanged for several months and is easily soluble in water. It must be noted that 4,4′-diaminodiphenylsulfone itself is insoluble in water, sodium bicarbonate or in any alkaline solution. Analytical and identity tests of diasone indicate that its structural formula is as follows:



On analysis, the nitrogen and sulfur values show that the product, which comes out of the reaction mixture, represents 92–93% of the substance corresponding to the above formula. The balance is accounted for by uncombined sodium formaldehyde sulfoxylate and sodium sulfate. Biological considerations call for experiments concerning the strength of linkages between the amino groups and sulfoxylate. When diasone is taken orally, it may be acted upon by the acid of the gastric juice, which ordinarily contains about 0.4% to 0.5% hydrochloric acid. Tests proved that 0.4% hydrochloric acid at 37° does not in the space of 2 hr., cause the splitting off of the formaldehyde sulfoxylate or the liberation of diaminodiphenylsulfone. Acetic acid or carbon dioxide does not change the product, except to cause its precipitation from alkaline solutions. The precipitate redissolves again on adding alkali.

The oxygen in the air, as seen from experiments detailed by us, slowly oxidizes diasone. Dissolved in water and exposed for several hours to a stream of air, free of carbon dioxide, the product is gradually precipitated because of the increasing acidity. On addition of alkali, most of the precipitate redissolves, leaving a small amount of insoluble residue. On prolonged exposure to the air, more of the alkali-insoluble substance is formed. In all of these operations 4,4′-diaminodiphenylsulfone remains attached to the formaldehyde sulfoxylate. The proof lies in the fact that precipitates formed by the hydrochloric acid, acetic acid or carbon dioxide are easily redissolved by the addition of alkali. Being insoluble in alkali, diaminodiphenylsulfone would not redissolve if it were split off. Furthermore, had the product of oxidation contained free diaminodiphenylsulfone, it would have been totally or partially soluble in acid. In reality it is insoluble in acid and shows only traces of the free diaminodiphenylsulfone. The fact that diasone can be diazotized and the diaminodiphenylsulfone quantitatively estimated by the Bratton and Marshall method (3) indicates that both amino groups are set free by nitrous acid and thereupon form a diazo product.

That the combination of the diaminodiphenylsulfone and sodium formaldehyde sulfoxylate in diasone is a firm one and can withstand the splitting effect of various physiological agents is shown in its chemotherapeutic behavior as contrasted with that of sulfone. Administered by mouth, diasone is approximately 20 times less toxic for mice and seven times less toxic for rabbits than the diaminodiphenylsulfone. While the toxicity is greatly diminished, the therapeutic effect of diasone in streptococcic and pneumococcic infections in mice is not decreased in proportion. The chemotherapeutic index, *i. e.*, the ratio of toxicity to therapeutic effect, is more favorable in diasone than in 4,4′-diaminodiphenylsulfone.

EXPERIMENTAL

Method of Preparation.—Dissolve 20 Gm. *p,p′*-diaminodiphenylsulfone in 800 cc. warm specially denatured alcohol, formula 3-A (consisting of 5 parts methyl alcohol and 100 parts ethyl alcohol). Then add 24 Gm. finely powdered sodium formaldehyde sulfoxylate and reflux on a steam bath. The sulfoxylate gradually is consumed and after about 3 hr. some precipitation takes place. Reflux a total of 5 hr. Cool quickly to 15° C. and maintain at this temperature for 1 hr. Cool, filter and wash with cold 3-A alcohol. Dry in vacuum desiccator. Forty grams = 95% of white amorphous powder; m. p. melts at 263–265° C. with decomposition after losing water at 100–110° C.

Anal. Calcd.: N, 5.38, S, 18.46, moisture 13.84. Found: N, 4.85, S, 18.00, moisture 12.00.

Easily soluble in water, slightly soluble in alcohol and insoluble in most of the organic solvents.

Effect of Oxygen of the Air.—Dissolve 1 Gm. diasone in 100 cc. water (*p*H about 6). Bubble air, which has first been passed through two packed columns of soda lime to eliminate carbon dioxide, into the solution for 6 hr. After 30–45 min. *p*H is approximately 4 and solution is now cloudy (this can be cleared by addition of some dil. NaOH). After 1½ to 2 hr. *p*H is below 4 and solution is now milky. At the end of 6 hr., *p*H is 3 and solution contains a precipitate. Add dilute sodium hydroxide to *p*H 8 and filter through a weighed crucible. The precipitate, after washing with water and drying, weighed 0.015 Gm. which corresponds to 1.5%.

The above precipitate is insoluble in dilute hydrochloric acid, acetone and alcohol and is not diaminodiphenylsulfone.

Effect of 0.4% Hydrochloric Acid.—One gram of diasone treated with 100 cc. 0.4% hydrochloric acid at 37° C. for 3 hr.

5 Min.—Sample gives clear solution on addition of dilute sodium hydroxide to *p*H 8.

1 Hr.—Sample gives solution with faint haze on addition of dilute sodium hydroxide

2 Hr.—Small amount of precipitate remains undissolved on addition of dilute sodium hydroxide.

3 Hr.—Precipitate does not redissolve on addition of dilute sodium hydroxide.

At the end of 3 hr., filter and wash with water, alcohol and ether.

Weight of precipitate 0.260 Gm. Insoluble in acetone, alcohol, very slightly soluble in dilute hydrochloric acid (faint diazo reaction), on boiling with dilute hydrochloric acid, cooling and diazotizing very strong color reaction obtained. Free diaminodiphenylsulfone found in the filtrate amounted to less than 1%.

REFERENCES

(1) Callomon, F. F. T., *Am. Rev. Tuberc.* 52 (1943), 97.

(2) Feldman, W. H., Hinshaw, H. C., and Moses, H. E., *Arch. Path.*, 36 (1943), 64.

(3) Bratton, A. C., and Marshall, E. K., Jr., *J. Biol. Chem.*, 128 (1939), 537.

Experiments on the Growing of Mydriatic Drug Plants*,†

A. F. Sievers and M. S. Lowman

Among the plants furnishing crude drugs of importance that are adapted to cultivation in the United States the mydriatic drug plants, belladonna, henbane and stramonium, have been of particular interest with respect to their possibilities as crops in this country. In normal times the nation's requirements of belladonna have been obtained entirely from Europe. Most of the henbane is also thus secured, although this plant grows wild in the Rocky Mountain states where the drug is collected when its market value is sufficiently high. Stramonium is likewise obtained through imports and collection from plants growing wild. Accordingly, when conditions arise which prevent or interfere with the importation of these drugs their production in this country becomes necessary to furnish all or part of our requirements. In the case of belladonna all of our needs must be obtained through domestic cultivation, but henbane and stramonium need to be grown only in sufficient amount to supplement supplies obtained from the wild plants.

The experiences with these plants as domestic crops have indicated the problems faced by growers unfamiliar with their requirements and have shown that they probably cannot be profitably grown in competition with foreign sources of supply. Consequently their introduction has been limited to those periods when the higher market values of their products provided sufficient inducement to growers. Such conditions existed during the first World War[1] and again during the present war the need for domestic production arose with the result that belladonna and henbane were again grown in 1942 to an extent which appears to have been adequate for our present needs.

The Division of Drug and Related Plants of the Department of Agriculture had an active part in planning such a growing program and following it through. The problem of getting an adequate supply of satisfactory seed of these plants caused some concern. The seed offered in the trade was in some cases of unknown origin which made it uncertain whether it was derived from types or strains of the plants that would produce satisfactory crops. Although this question could not be entirely settled in time for the 1942 planting, experiments were undertaken to obtain information regarding it for future use. There was also need for information concerning the effects of drying these crops in artificially heated drying rooms on color and alkaloid content. The difficulty of satisfactorily drying such herbaceous plants without recourse to artificial means has long been recognized. Experiments were therefore undertaken in 1941 to obtain basic information along that line but since the results were not entirely conclusive the experiments were continued in 1942.

The present paper presents the results of the two lines of investigation: (1) the comparison of plants of belladonna and henbane from different seed sources with regard to productiveness and quality, and

* Received August 28, 1943 from the Division of Drug and Related Plants, Bureau Plant Industry, Soils, and Agricultural Engineering, Agricultural Research Administration, United States Department of Agriculture.

† The alkaloidal assays required in connection with these experiments were made by Frank Rabak and J. W. Kelly, Division of Drug and Related Plants.

[1] Sievers, A. F., THIS JOURNAL, 29 (1940), 408.

(2) effects of various temperatures and methods of handling on the rate of drying and the quality of belladonna, henbane and stramonium produced.

COMPARISON IN EARLY STAGES OF SEEDLINGS GROWN FROM SEED FROM VARIOUS SOURCE

Seed of belladonna from six sources and seed of henbane from eleven sources were used. The origin of the seed in each case so far as known is discussed later and is shown in connection with the tabulated data in Tables II and IV. The seed was sown in flats in steam-sterilized composted sandy loam in the greenhouse on February 9. The temperature was maintained at 65–70° F. at night and 75–85° F. in the daytime. The seedlings were later transplanted to sterilized soil in 2 x 2 x 3-inch plant bands in which they were kept in the greenhouse until May 7 when they were transplanted to the field. During this period the seedlings from the several lots were under close observation to note differences between the several lots from each in botanical characters, growth rate, resistance to disease, etc.

Belladonna.—The belladonna seedlings from seed lot No. 6 were distinctly different from those of the other five lots in regard to growth habit (Fig. 1). They grew more rapidly after the first month and when set in the field had reached a height of 8 to 14 inches with a single spindly stem. All the others were about 6 to 10 inches tall and less spindly, except those of lot No. 3 which were less vigorous, grew slower and were only 3 to 4 inches tall. There were not enough of these plants to include them in the replicated field plantings. Apparently the seed from this lot was inferior both in viability and in the vigor of the seedlings produced. The total germination of the seed from the six lots was 70, 60, 25, 85, 80 and 85%, respectively.

Henbane.—Among the several lots of henbane the plants from No. 5 were soon observed to be an annual type. It produced a flower stalk at a very early stage, and all the seedlings were in bloom before the plants were transplanted to the field. Even some of the seedlings left in the seed flat produced bloom when only 2 inches high. After transplanting little growth was made and the plants soon matured and died. The plants from lots Nos. 3 and 9 also turned out to be annual types, but they did not differ in their general appearance from the biennial types up to transplanting time except that a single plant in lot No. 9 produced a few flowers while in the greenhouse (Fig. 1). The plants in this lot developed good rosettes of leaves but when transplanted these soon dried up and only large flower stalks were formed. Those from lot No. 3 developed tall flowering stalks with small leaves. On the basis of their growth under the conditions which prevailed all three of these annual types would be undesirable for commercial culture on account of low yields. However, lots Nos. 5 and 9 contained 0.063 and 0.056% of alkaloids, respectively.

Susceptibility to Disease.—The belladonna and henbane seedlings were equally susceptible to damping off fungi in the flats and later in the plant bands, caused mainly by species of *Pythium* and *Rhizoctonia*, regardless of the source of the seed.

COMPARISON OF PLANTS IN THE FIELD

Identical procedures were used for the replicated field plantings of these several lots of belladonna and henbane. There were six replicated plots of each lot planted with six rows of six plants each spaced 3 x 3 feet. The field was not very uniform with respect to soil, both as regards fertility and physical conditions. The effect of such differences could be clearly seen in several cases in the growth of the plants, but it is believed that on the whole the number of replications were sufficient to reduce to a minimum the effects of such influences on the data obtained. The growth habits and general appearance of the plants in the several lots were carefully noted up to the time of the first harvest.

Belladonna.—The belladonna plants in the field from seed lots Nos. 1, 2, 4 and 5 were very similar in appearance as was the case in the greenhouse (lot No. 3 did not furnish sufficient plants to be used in the replicated plantings), but those from lot No. 6 were different enough to suggest a different species. They grew tall; had heavy, usually non-branching stems; narrow, pointed, light green leaves, and yellow flowers. The ratio of leaf to stem was definitely less than in that of the other lots and flowers developed later. After careful examination a taxonomist declared it to be *Atropa acuminata* Lindl., an Indian species.[2]

Although the other lots did not show any difference in the early seedling stage, it was believed likely that distinct type differences might develop later, particularly in the size of leaf, profusion of flowering and fruiting, which would affect yield and alkaloid content. The seed of lots Nos. 1 and 2, although obtained from the same individual, were not received at the same time and were, therefore, handled separately throughout. Later it was reported to have come from the same supply. The contributor of these two lots originally obtained seed for domestic planting from plants in Germany, selected for vigor, large leaf and possibly high alkaloid content. Lots Nos. 4 and 5, though of less definite origin, represent types that have long been cultivated in this country and furnished a drug of satisfactory quality well above the Pharmacopœia requirement of alkaloid content. Early in July, shortly before the harvesting began and when the plants were beginning to bloom, certain measurements were made to permit comparison of the plants from lots Nos. 1, 2, 4 and 5, with respect to stalk growth and leaf size. The complete data need not be recorded here but average minimum and maximum length of the stalks and the average width and length of the largest leaves observed in the six replications of these four lots are compared in Table I.

TABLE I.—COMPARISON OF GROWTH MEASUREMENTS OF BELLADONNA PLANTS FROM SEVERAL SEED LOTS SHORTLY BEFORE THE HARVESTING OF THE FIRST CROP ON JULY 15

Lot No.	Average Length of Stalks		Average Dimensions of Largest Leaves	
	Minimum, Inches	Maximum, Inches	Width, Mm.	Length, Mm.
1	10.8	22.5	101	221
2	11.8	23.0	109	217
4	8.5	21.3	104	211
5	8.0	21.0	105	215

It appears from the data in Table I that the plants from seed lots Nos. 1 and 2 made slightly more growth than those from lots Nos. 4 and 5 but differences between replicated plots of any one lot were in some cases as great as those here indicated between lots. The differences in leaf size between plants of the several lots is too small to be significant.

[2] Since this paper was written, the results of a study of this species grown in England has been published by C. E. Corfield, E. W. Kassner and E. Collins under the title, "Indian Belladonna," in *Quart. J. Pharm. Pharmacol.*, 16 (1943), 108.

In general the differences are not sufficient to serve as type distinctions, and the plants did not display any botanical differences. There is some indication that lots Nos. 1 and 2 might be slightly more productive. The data on yields as shown in Tables II and III should be consulted in connection with the foregoing growth comparisons.

Henbane.—The origin of the several lots of henbane seed and the general condition of the plants when they were harvested on July 13 are indicated in Table IV. As already mentioned, lots Nos. 3, 5 and 9 were annuals and all three failed to produce satisfactory crops, hence yield data of these are not recorded here. Seed of lot No. 5 was also sown directly in the field late in May and produced flowers when only 3 to 6 inches high and made very little leaf growth. It appears, however, that lots Nos. 3 and 9 may make fairly good leaf growth on the flowering stem.

promptly from the stems remaining and from below ground level. A second crop from these three rows of plants cut high was obtained on August 17 and a third on September 29 from two of the rows, the third row having been used to furnish material for special drying experiments.

The plants in the two rows cut close to the ground on July 15 were definitely injured by this procedure. A large proportion of them died, and on the others new growth was slow. A second crop could not be cut until September 1. Only yield data were obtained. Data on the yields and quality of material obtained by the two procedures are assembled in Tables II and III.

Henbane.—Five rows of henbane in each of the replicated plots were harvested, one row being left for general comparison and observation on winter survival and for seed production the next year. These five rows included 30 plants set out in each

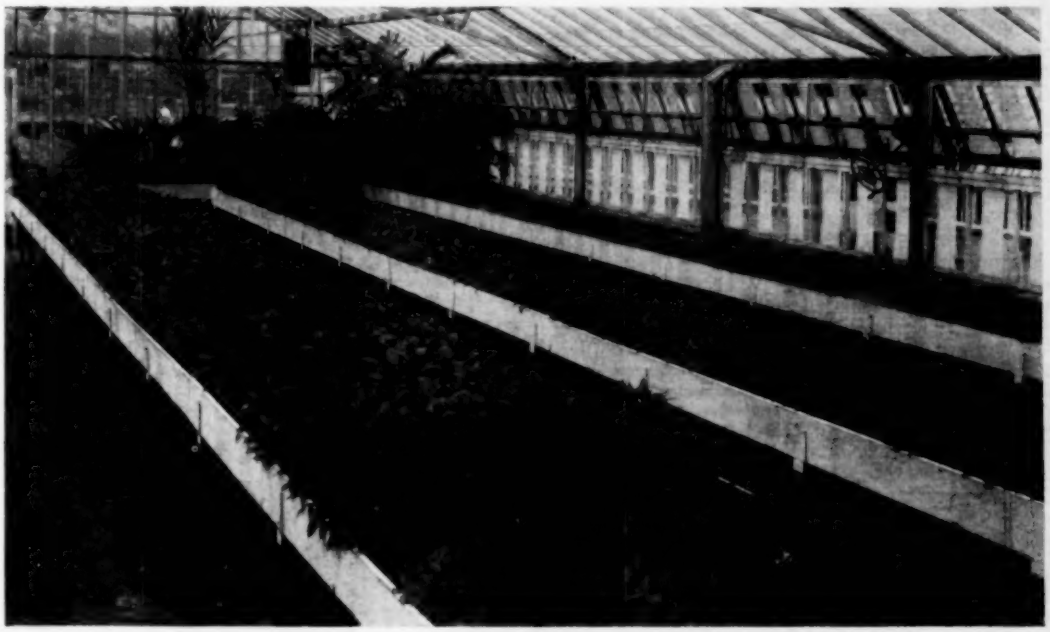


Fig. 1.—Belladonna and Henbane Seedlings Ready to Transplant.

Belladonna lot No. 6, in center foreground later identified as an Indian species, *Atropa acuminata*, is distinguished by its larger size from lots 1 to 5 beyond it, which all look alike. The henbane in the foreground, on the same bench, is an annual type (No. 9) which grew rapidly, but the leaves dried up when the flowers developed immediately after transplanting. On the bench beyond are other lots of henbane.

HARVESTING

The harvesting plan followed for the belladonna and the henbane differed. In all plots one row was left to permit observation of the plants throughout the season and provide information on winter survival of the several lots when undisturbed. The remainder of the plantings were intended to furnish information on various harvesting procedures. It was expected that the henbane could be harvested only once or twice during the season. In the case of belladonna, however, it was sought to obtain information on the effects of various factors on yields, quality and survival.

Belladonna.—On July 15 three rows were cut 6 to 8 inches from the ground while two rows were cut no more than 3 inches from the ground. A majority of the plants cut at the lower level died, but those cut at the higher level developed new growth

plot or a total of 180, but a few had in all plots failed to become established or died subsequently. All the leaves, except the small undeveloped ones, were pulled from the plants. The loss of plants after the leaves were harvested was extremely heavy in all lots. Comparatively few plants survived. This loss was probably due to some extent to excessive rains and inadequate drainage. Most of the plants remaining after several weeks developed new leaves slowly, and the leaves were yellowish and rough, suggesting unfavorable growth conditions. Many also showed symptoms of a virus disease. The removal of the leaves could not have been responsible for the loss of the plants because those in the rows that were not harvested likewise died by fall. Data on yields and quality of the material harvested are shown in Table IV.

TABLE II.—COMPARISON OF PLANTS OF *Atropa belladonna* GROWN FROM SEED FROM VARIOUS SOURCES AND OF *Atropa acuminata*, AND DATA ON YIELD AND QUALITY OF CROP WHEN CUT 6 TO 8 INCHES FROM GROUND

Plant	Seed Lot No.	Source of Seed	Germination (Seed Planted Feb. 9), %	Size and Condition of Seedlings When Transplanted to Field May 6	Harvesting Data[a] Date	Dry Herb, Gm.	Plants Harvested	Dry Herb per Plant Gm.	Stems more Than 10 Mm. diam. %	Calculated per Acre Yield of Dry Herb Based on Actual Stand of Plants, Lbs.	Based on Full Stand of Plants, Lbs.	Alkaloids[b] Leaves, Tops and Small Stems %	Large Stems %	Color of Dried Drug	Botanical Comparisons
Atropa belladonna	1	From domestic grown plants; original seed from plants in Germany selected for vigor, size of leaves and other desirable qualities	60–70 in 36 days	Rapid growth; vigorous, 6 to 10 inches	7–15	2596	107	24.26	6.8	257	259	0.620	0.388	Dull greenish brown	The seedlings obtained from seed lots Nos. 1, 2, 3, 4 and 5 were very similar in the early stages. Later those from No. 3 were retarded in growth and less vigorous. In the field the plants from the other four lots showed no botanical differences. Apparently these as well as the few from No. 3, which were set in another plot, are all of the form typical of *Atropa belladonna*
					8–17	3645	106	34.39	4.3	360	367	0.586	...	Greenish brown	
					9–29	1490	68	21.91	None	221	234				
					Total	7791				838	860				
Atropa belladonna	2	From domestic grown plants; original seed from plants in Germany selected for vigor, size of leaves and other desirable qualities	50–60 in 36 days	Rapid growth; vigorous, 6 to 10 inches	7–15	3426	107	32.02	8.4	339	342	0.622	0.293	Dull greenish brown	
					8–17	2741	105	35.62	3.2	369	380	0.659	..	Greenish brown, somewhat dark	
					9–29	2245	70	32.07	None	332	342				
					Total	9412				1040	1064				
Atropa belladonna	3[c]	Of Yugoslavian origin; probably obtained from drug imported from that country	25 in 44 days	Very slow growth; lacking in vigor; 3 to 4 inches	8–17	..	..	..	..	..	..	0.566	..	....	
Atropa belladonna	4	From domestic grown plants; earlier history unknown. Seed sold in open market	85 in 26 days	Rapid growth, vigous, 8 to 10 inches	7–15	2207	104	21.22	8.5	218	226	0.559	0.330	Dull greenish brown	
					8–17	4032	104	38.77	4.5	403	418	0.517	...	Greenish brown	
					9–29	2320	72	32.22	None	344	344				
					Total	8542				965	981				
Atropa belladonna	5	From domestic grown plants under cultivation for many years; originally selected for alkaloid content	75–80 in 34 days	Rapid growth; vigorous; 6 to 10 inches	7–15	2274	104	21.87	4.3	223	232	0.595	0.270	Dull greenish brown	
					8–17	4364	107	40.80	2.5	432	436	0.503	...	Greenish brown	
					9–29	1970	71	27.75	None	292	296				
					Total	8601				947	964				
Atropa acuminata	6	From India	85 in 29 days	Very rapid growth; spindling; 8 to 14 inches	7–15	2088	106	19.70	18.4	206	210	0.380	0.138	Dull greenish brown	These plants are distinctly different from those above. They grow taller, have much higher proportion of large stems, more pointed leaves of a lighter color and yellow flowers
					8–17	2397	104	23.05	25.0	237	246	0.316	...	Greenish brown	
					9–29	1148	62	18.52	[d]	170	197				
					Total	5633				613	653				

[a] Plantings in all cases consisted of six replications of plots of 36 plants set in six rows of six plants each spaced 3 × 3 feet. At the first harvest three rows were cut 6 to 8 inches above the ground, and the total yields recorded represent the yields from all six replications. The second harvest was made from the same rows. The third harvest was made from only two of these rows, hence the smaller number of plants and the lower total yields as indicated. The yields per acre are calculated on the basis of the yield per plant and a complete stand spaced 3 × 3 feet and also on the basis of the stand actually obtained. The yields shown are of the U. S. P. product exclusive of the larger stems removed.

[b] The drug designated as leaves, tops and small stems represents the harvested crop after the large stems had been removed.
[c] Insufficient plants to include this in the replicated plantings.
[d] Not determined, but very small percentage of the stems were over 10 mm. in diameter compared with the two previous cuttings.

TABLE III.—COMPARATIVE DATA ON YIELD AND QUALITY OF *Atropa belladonna* GROWN FROM DIFFERENT LOTS OF SEED AND OF *Atropa acuminata* WHEN CUT 3 INCHES OR LESS FROM THE GROUND

(For descriptive details of the plants see Table II)

Plant	Seed Lot No.	First Harvest, July 15					Alkaloids		Second Harvest, September, [d]					Calculated per Acre	Seasonal Yields
		Dry Herb.[a] Gm.	Number of Plants	Yield per Plant, Gm.	Stems More Than 10 Mm. Diam., %	Calculated Yield per Acre,[b] Lb.	Herb without Large Stems,[c] %	Large Stems, %	Dry Herb, Gm.	Number of Plants	Yield per Plant, Gm.	Stems More Than 10 Mm. Diam., %	Calculated Yield per Acre, Lb.	On Basis of sufi Stand at Both Harvests, Lb.	On Basis of Actual Stand at Both Harvests, Lb.
Atropa belladonna	1	2394	71	33.71	15.23	360	0.605	0.256	579	29	19.96	None	213	573	438
Atropa belladonna	2	3387	72	47.04	14.96	503	0.624	0.212	1129	49	23.04	None	246	749	670
Atropa belladonna	4	1980	70	28.29	15.02	302	0.527	0.223	808	42	21.38	None	228	530	427
Atropa belladonna	5	2159	70	30.84	13.60	330	0.478	0.211	920	41	22.44	None[c]	240	570	457
Atropa acuminata	6	2020	70	28.86	23.9	308	0.353	0.150						308	299

[a] Total weight from six replications.
[b] This is the yield of herb containing no stems more than 10 mm. in diameter.
[c] Same material as referred to in [b].
[d] No assay wgs made of this material.

DRYING

Belladonna.—That portion of the material harvested as the first crop of belladonna obtained from the three rows cut 6 to 8 inches high was spread on the wooden floor on the second story of a large building where there were considerable light and good ventilation. It was turned twice daily for several days, then daily until partially dry. Fourteen days were required to get it into such condition that it could be stored in tightly filled bags. That obtained from two other rows cut much closer to the ground was tied on laths as is sometimes done with tobacco and the laths then placed side by side on supporting cross pieces about 5 feet above the floor. The laths were so placed that when the suspended drug wilted adequate circulation of air through the material was possible. When so handled the drying was accomplished in 12 days. A second crop was obtained on August 17 from the three rows previously cut 6 to 8 inches above the ground and again cut in that way. All this was tied on laths as described and required about 14 days to dry. During both periods the weather was sultry with high humidity. The drying could not have been completed in the time indicated if a large amount of the succulent material had been placed in the building. Therein lies the difficulty of drying the crop commercially without use of artificial heating, especially if good drying weather does not prevail. The moisture loss in most cases was between 85 and 88%, being highest in the second crop. After placing the dried drug in bags it was allowed to stand for a while and then placed in a drying chamber for about 1 hr. at 120° F. to get it brittle, after which the stems above 10 mm. in diameter were separated and the percentage of these determined. Each lot was then ground coarsely, thoroughly mixed and a representative portion reserved for subsequent further grinding to furnish a sample for assaying. Two products were thus obtained in each case, one consisting of leaves, tops and small stems not exceeding 10 mm. in diameter, and the other consisting exclusively of stems of varying diameters in excess of approximately 10 mm. Such samples were obtained, representing six replications of each of the five lots harvested in the way described. A third crop cut on September 29 from two of the same three rows previously twice cut 6 to 8 inches from the ground was weighed to secure yield data and then used for special drying experiments. However, the yield data are included with all the related data in Table II pertaining to the rows thus harvested.

Henbane.—The henbane was all dried on trays. The leaves were washed to remove most of the adhering soil, then spread in screen-bottomed trays about 1 x 3 feet and placed in an attic room. The leaves from some of the lots were very large and drying proceeded very slowly in the humid, rainy weather that prevailed for several days. When clear weather occurred, the trays were set outside on racks in the sun for a day and then returned to the attic. About 2 weeks after the general harvesting composite samples were collected from all the plants in the remaining row and likewise dried on screens on the top floor of a building. Inasmuch as these were small samples and less humid conditions prevailed, the drying was accomplished in less time.

YIELD AND QUALITY OF HERB

Belladonna.—An inspection of Table I for a comparison of lots Nos. 1, 2, 4 and 5 shows that while the seed from lots Nos. 1 and 2 were later found to be from the same source, the calculated per acre yield from No. 2 was the highest and that from lot No. 1 the lowest in the group. The more favorable locations of one or more of the replicated plantings

TABLE IV.—COMPARISON OF PLANTS OF *Hyoscyamus niger* GROWN FROM SEED FROM VARIOUS SOURCES

Seed Lot No.	Source of Seed	Germination (Seed Planted Feb. 9), %	Size and Condition of Seedlings When Transplanted to field May 6. Rate of Growth	Harvesting Data[a] (Date of Harvesting, July 13) Dry Herb, Gm.	Plants Harvested	Dry Herb per Plant, Gm.	Dry Herb per Acre	Alkaloids[b] %	Color of the Dried Drug	Botanical Comparisons
1	Open market	60 in 15 days	Vigorous; 4 to 6 inches	4471	177	24.27	279	0.058	Brown	Biennial; several types, some more vigorous than others; leaves 3 to 6 inches broad; 6 to 16 inches long
2	From wild plants in Montana	85 in 14 days	Quite rapid; 6 to 7 inches	3707	172	21.56	238	0.052	Green	Biennial; uniform in type with long tapering smooth leaves 2 to 5 inches wide and 6 to 16 inches long
3[c]	From crude drug imported from Hungary	10 in 20 days	Slow; 3 to 4 inches	...	...	...	...	...	...	Annual; 18- to 24-inch flowering stalk; small leaves along stalk; leaf yield less than in biennial types
4	From pharmaceutical manufacturers. History unknown	85 in 29 days	Vigorous; 4 to 6 inches	3782	163	23.26	257	0.060	Brown	Biennial; long crinkly leaves 3 to 7 inches broad, 12 to 18 inches long
5[d]	From northern Yugoslavia	30 in 23 days	Slow; poor; 2 to 3 inches	...	...	...	...	0.063	...	Annual; 6 to 8 inches high; blooming when set in field. Very few leaves produced
6	From wild plants in Montana	75 in 27 days	Slow; poor; 3 to 4 inches	2858	163	17.71	196	0.086	Green	Biennial; smooth, thin, deeply serrate leaves 2 to 6 inches wide, 8 to 18 inches long; dark green color; spread close to ground
7	From domestic cultivated plant	85 in 14 days	Vigorous; 5 to 8 inches	6735	178	37.83	418	0.051	Green	Biennial; very vigorous with long, tapering, silvery pubescent leaves, 3 to 6 inches wide, 12 to 22 inches long; uniform productive type
8	From domestic grown plant. Seed originally from Chungtien Plateau, China	60 in 15 days	Fair; 4 to 6 inches	12565	178	70.68	779	0.072	Dark brown	Biennial; large, nearly oval, light green, very crinkly, and nearly glabrous, short petioled, more or less upright leaves 6 to 12 inches wide and 15 to 22 inches long; very productive type
9[e]	From domestic grown plants from seed originally from Germany	90 in 10 days	Very vigorous; 6 to 8 inches	...	...	...	...	0.056	...	Annual; vigorous, producing good rosette of leaves before flowering; heavy flower stalk with many large seed pods
10	From commercial source. Probably from wild western plants	85 in 20 days	Fair; 4 to 6 inches	5094	173	29.46	325	0.079	Brown	Biennial; leaves rather variable, crinkly, light green 6 to 10 inches wide and 16 to 20 inches long. Fairly productive
11	From plants grown in New Hampshire	85 in 14 days	Vigorous; 5 to 8 inches	5575	171	32.60	360	0.045	Green	Biennial; very similar to No. 7 in leaf character but has smaller leaves and is less productive

[a] Plantings in all cases consisted of six replications of plots of 36 plants set in six rows of six plants each spaced 3 × 3 feet. When harvested one row was left in all cases for later observation, survival, etc., and five rows were cut. The yields from each plot were recorded but the data here shown are the totals for the six replications.
[b] Represents the crop from each lot from six replications.
[c] Insufficient plants to include in replicated plantings.
[d] This annual type bloomed before transplanting. Made practically no growth in field.
[e] This annual type made good growth of leaves in greenhouse but when transplanted these dried up and only flower stalks developed.

of lot No. 2 in the field, as indicated by the relative size of the plants, are at least partly responsible for this difference. When these two lots are grouped together, the average calculated per acre yield becomes 962 lb. as compared with 981 and 964 in the case of lots Nos. 4 and 5, respectively. It seems, therefore, that the seasonal yield from the plants grown from these several seed lots when thus harvested may be expected to be about the same. Lot No. 2 differed from the others also in that each of the three cuttings yielded more nearly the same whereas in the other lots the second cutting produced the most by a considerable margin. The Indian species, lot No. 6, is clearly much less productive. The percentage of stems in excess of 10-mm. diameter in lots Nos. 1, 2, 4 and 5 is in all cases lower in the second than in the first cutting and no stems needed to be removed from the third cutting. In lot No. 6 the percentage of large stems was extremely high and was higher in the second than in the first cutting.

The acre yields are calculated, based on full stands and also based on the actual stands in the combined six plantings. They are lower than would be the case in most commercial plantings because somewhat closer spacing in the row than 3 feet would usually be permissible, although the yield increase would probably not be in the same proportion. Inasmuch as a full stand of plants is seldom realized in commercial plantings the yields calculated on actual stands are considered better indications of what may be expected under practical farming conditions. The yields obtained when the cuttings were made close to the ground are shown in Table III. In all cases the total yields for the season were much less than when the cuttings were made 6 to 8 inches above the ground (Table II). The reduction in seasonal yield resulting from this procedure is due to the large loss of plants resulting from it and the much slower development of new growth on the surviving plants as indicated by the fact that only two harvests were possible instead of three. Here again lot No. 2 gave the highest yield, due in part to the more favorable location of at least one of the replicated plantings as previously stated. The alkaloid content of the first crop cut 6 to 8 inches from the ground, after the large stems had been removed, and of the stems alone, is about the same as that of comparable material obtained by cutting the plants closer to the ground. Lots Nos. 1 and 2 are somewhat superior and lot No. 6 is the poorest. Only the large stems of lots Nos. 2, 5 and 6 had an alkaloid content lower than the 0.3% required for belladonna herb by the Pharmacopœia. The drying of the herb as described did not result in the much desired uniform green color. The color was more a greenish brown and greenish gray. No doubt the humid and rainy weather that prevailed was largely responsible. The results emphasize the need for special equipment for drying the crop if a product with good color is desired.

Henbane.—The description of the plants in the several lots and the yield data in Table IV clearly indicate the wide differences in the biennial types. Lot No. 8, which originated in China, was outstanding for comparative size of leaf and therefore per acre yield, producing at the rate of 779 lb. The next best producer was lot No. 7, also characterized by large leaves, which yielded at the rate of 418 lb. The original source of this is unknown, but it had been grown from year to year prior to 1940 in near-by Arlington County, Virginia. The yields would have been substantially larger in all cases if the plants had been spaced closer in the row. Moreover, under different conditions more favorable to the survival of the plants new growth that would provide a second crop would further increase the yield.

The alkaloid content of the large lots from the general harvesting on July 13 varied considerably as shown in the table but in all cases was above the Pharmacopœia requirement. It will be noted that lot No. 8, which gave by far the largest yield, is high in alkaloid content and would, therefore, be an excellent type to grow unless further trials reveal undesirable characters not yet observed. Lot No. 7, also a good producer, is considerably lower in alkaloid content but well above the requirement. The smaller samples collected later from the reserved rows as referred to and which were dried with less difficulty were also assayed with results that compared favorably with those obtained with the large lots.

The color of the dried lots differed greatly. Some had a good green color while others were brown. There is no apparent reason why the drug from the several seed lots should differ in this respect. The poor color of lot No. 8 may be due to the large size of the leaves and its general bulkiness which makes it more difficult to handle on screens. In some cases the edges of the leaves turned black.

Yield and Quality of Belladonna, Henbane and Stramonium from Experimental Plots in 1941.—Some experiments were conducted in 1941 on light sandy soil with the three principal mydriatic drugs which furnished data on yields and quality, and the effects of certain drying methods which are recorded and discussed here in connection with the report on the investigation in 1942. The areas harvested were small and yields per acre calculated from the actual yields from the plots serve therefore only as approximate indications of probable commercial yields. All the data on yields are assembled in Table V. On the whole, the yields of belladonna compared well with those obtained in 1942, although the soil is quite different. The henbane designated as type 2, which is the same as lot No. 8 in the 1942 experiments, yielded substantially more than type 1 (lot No. 7 in 1942), but in this case two crops were obtained. If the plants in 1942 in the replicated plots had not been destroyed by excessive rain and had made the same relative second growth the seasonal yields would undoubtedly have exceeded those obtained in 1941.

Practically all of the henbane plants died late in fall or during the winter. A virus disease, which was identified with reasonable certainty as that which causes tobacco mosaic, became widely disseminated in the plot. This was probably the same as that which attacked the plants in 1942 but did not develop so early in the season in 1941. The leaves were picked in two different ways. One way was to pull them off with the entire petiole whereas in the other they were pinched off, leaving most of the petiole on the plant. It was thought that the latter method would be less injurious to the plant, but no difference was observed in the rate or quantity of the new growth which developed. Since the plants handled in both ways died, it appears that leaving the petioles on the plants was of no value. Moreover, these petioles contain as much or more alkaloids than the leaf itself, hence may well be included in the crop. If the plants are to be dried by being hung up in bunches, this can only be done if the leaves are pulled off with the entire petiole attached.

The experience with henbane in both years strongly indicates that under the conditions that prevailed it must be grown as an annual crop by such methods as will produce the largest yields from a single or several cuttings. This is certainly true in localities where virus diseases attack the plant in late summer, and this type of disease has been reported in numerous localities widely separated. No doubt cultivating implements and harvesting of the

leaves spread the disease through the field. Perhaps if the plants are not harvested the first year the disease will not develop, and a heavy crop can be obtained in the second year. If that is the case, the proper procedure must be determined by the relative net returns.

The yields of stramonium obtained were high, but the cost of collecting the leaves or the young tops, as was done in this case, would be prohibitive unless the market value of the drug is far above normal. The seed was sown directly in the field in two long rows. A good stand was obtained in a short time, and when the seedlings were about 6 inches high, the plants were thinned to stand 3 to 4 inches apart in one row and 6 to 8 inches in the other. For some time there was little noticeable difference in the appearance of the two rows, but later the plants with the wider spacing grew taller and produced about 7% more material. The alkaloid content of the drug obtained was much higher than the 0.25% required by the U. S. Pharmacopœia.

like tobacco is handled, at atmospheric temperatures cannot be depended upon for the best results in regions where humid and rainy weather may occur. In such regions, and preferably in all regions, the use of artificially heated rooms or other equipment or facilities in which heat may be maintained with proper movement of air is essential for satisfactory drying of these crops. It is important, therefore, to know what effects certain conditions have on the drug when so handled. Information concerning the temperature that may safely be used and the maximum time the drug may be exposed to it is required to determine the details of the equipment needed.

An examination of the data in the tables permits the conclusion that the alkaloid content is not materially affected by rapid drying at high temperatures. It is very difficult to prepare samples for analysis from several comparable lots of the herb in freshly cut condition when stems are present. Moderate differences in alkaloid content of such samples could be due to this fact. This must be

TABLE V.—YIELDS OF BELLADONNA, HENBANE AND STRAMONIUM FROM EXPERIMENTAL PLOTS ON LIGHT SANDY SOIL IN 1941

Crop	Description and Condition of Herb Harvested	Date of Harvest	Area Harvested, Acres	Yield of Dried Drug From Area Harvested Lb. Crop	Yield of Dried Drug From Area Harvested Lb. Season	Yield of Dried Drug Calculated per Acre Lb. Crop	Yield of Dried Drug Calculated per Acre Lb. Season
Belladonna	Tops including leaves, small stems and some flowers cut 6 to 8 inches above ground						
	First crop	July 24	0.015	6.07	11.00	400	726
	Second crop	Sept. 3	0.015	4.93		326	
Henbane	Leaves pulled off close to crown						
	Type 1[a]						
	First crop	Aug. 17	0.033	16.29	24.22	493	733
	Second crop	Sept. 22	0.033	7.93		240	
	Type 2						
	First crop	Aug. 17	0.0165	11.01	16.54	666	1001
	Second crop	Sept. 22	0.0165	5.53		335	
Stramonium	Tops including leaves, small stems and some flower buds						
	(1) Plants spaced 8 to 10 per foot in row	Aug. 20[b]	0.0041	..	11.07	..	2679
	(2) Plants spaced 2 per foot	Aug. 20[b]	0.0041	..	11.49	..	2880

[a] Types 1 and 2 are the same as lots Nos. 7 and 8 in 1942 plantings. (See Table IV.)
[b] If harvested later the difference in yield between (1) and (2) would have been much greater because the less crowded plants grew considerably larger.

DRYING EXPERIMENTS

In 1941 some experiments were begun to determine the effects of various methods of drying on belladonna, henbane and stramonium. It was sought to determine to what extent, if any, rapid drying in special drying chambers at rather high temperatures affected the quality of the drug as compared with slow drying of similar material without the use of artificial heating. The results obtained are assembled in Table VI. Additional experiments along the same line were made with belladonna in 1942, details of which are given in Table VII.

The quality of these drugs is judged by dealers in crude drugs and manufacturers of the pharmaceutical products into which they enter, by their color and alkaloid content, both of which may be affected by methods of drying or curing. A third factor which affects quality, namely, the proportion of stems present, is also related to the problem of drying in that large stems require more time to dry than small stems and leaves and, therefore, make it necessary to expose the drug longer to the particular conditions used. It is a well-known fact that drying by spreading the herb on wooden floors or on trays, or by suspending it from laths or sticks borne in mind when the results are considered.

Belladonna.—In 1941 all the belladonna lots dried in the heated chamber showed about the same alkaloid content, although the temperatures used ranged from 122° to 194° F. (Table VI). The lot dried slowly on trays in 10 days (No. 1*a*), contained a somewhat lower percentage, but the difference can hardly be considered as indicating that the rapid drying increased the alkaloid content. In 1942 three temperatures were used in comparison with slow drying on wire-bottomed trays and by suspension from sticks, both of which required from 9 to 11 days (Table VI). Lot No. 12 dried at 130–131° F. and lot No. 11 dried at 165–167° F. with the two companion lots (lots Nos. 8 and 9) dried slowly, as indicated, are in direct comparison because all the material came from one general lot. The alkaloid contents of these lots do not differ greatly, indicating that the temperatures within the limits used and under the condition of these experiments do not affect the total alkaloid content. Lots Nos. 4, 5 and 6 represented material different from the above as indicated in the table. Two of these, lots Nos. 5 and 6, were dried slowly by the two procedures mentioned and lot No. 4 at 144–146° F. The alkaloid contents of these three lots are also in close agreement.

Additional drying experiments with belladonna were made in 1942 with special consideration of the effects of an initial moderate temperature followed by a higher temperature after partial drying as compared with a high temperature throughout the period. Inasmuch as only one drying experiment could be conducted at a time it was necessary to obtain fresh material from the field for each experiment. While such material was all obtained from the same planting, some differences in the several portions used may be expected because the proportion of stems of various diameters in such portions may have varied considerably. Referring to table VII it is noted that the variation in the alkaloid content is relatively small with the exception of lot No. 41. There is no obvious explanation for the considerably higher alkaloid content of this lot dried partially at 125° F. followed by a slightly longer period at 200° F. It would seem that if such procedure is noticeably beneficial, similar effects would have been obtained in the case of lot No. 42. In general, the results of these experiments are in accord with those obtained in 1941 and in the other experiments in 1942, indicating that as far as the alkaloid content of the drug is concerned belladonna may be dried at temperatures that will get the drug into condition for baling in less than 12 hr. if the proper movement and replacement of air is maintained in the drier. The effects of the various conditions of drying on the color of the dried herb do not permit any definite conclusions. In general a good green color is obtained when rapid drying is accomplished within 24 hr. The color is obviously adversely affected when a high initial heat is used as indicated by lot No. 43. Slow drying on screens or by suspension from sticks will result in a good color, if the weather is favorable and the drying can be completed in 10 days.

Henbane.—In the case of henbane the temperatures used in the experiments in 1941 were less extreme but here again the results suggest that drying at temperatures up to 178° F., the highest used, has no detrimental effect on the total alkaloid content (Table VI). However, the experiments were probably too limited in number to permit definite conclusions.

Stramonium.—In the experiments with stramonium in 1941, two kinds of material were used: (1) the tops consisting of leaves and stems from 3 to 6 inches long, removed from the plants with shears, and (2) leaves only, taken from all parts of the plants. In collecting the first-mentioned material it is impossible to prevent a varying proportion of stems in successively picked lots, and a moderate variation in the alkaloid content of such lots is therefore to be expected. On the whole, the samples of both types of material dried on trays and at a maximum temperature of 187° F. contained about the same percentage of alkaloids (Table VI). From this it may be concluded that such temperatures as in the case of belladonna are not injurious in that respect.

None of the lots had a good green color when dry. Most of them had a greenish brown or grayish green color.

WINTER SURVIVAL OF BELLADONNA

A serious problem of the belladonna grower is the tendency of the plant to die during the winter. Year-old plants in good condition start to grow early in the spring and generally produce larger yields than seedlings. This and the elimination of the propagation costs involved in starting the crop from seedlings make the second season crop from the previous year's field more profitable. However, experience everywhere has shown that when the plants are repeatedly cut during the first year and especially if they are cut near the ground or late in the fall, the percentage that survive the winter is too small to justify retention of the field. Certain factors have a direct bearing on the degree of survival and various procedures have been followed by growers to reduce winter killing. In the experiments with belladonna reported in this paper, some observations were made on that point that are worth recording at this time.

The belladonna grown from five seed lots in replicated plantings afforded opportunity to observe the effect of cutting and mulching on the survival of the plants. As stated, one row from each lot was not cut. Two rows were cut close to the ground, and in these most of the plants died soon thereafter. In three other rows the plants were cut 6 to 8 inches above the ground three times. Of these rows one was left unprotected in the winter, and two were mulched with about 4 inches of straw. The effects of these conditions and treatments are shown in Table VIII.

The data clearly indicate that under the conditions that prevailed: (1) if *Atropa belladonna* is not harvested at all, there is practically no winter killing; (2) if harvested several times, the last time late in the summer, and if no mulch is provided, there is hardly any survival; (3) if the late harvested plants are mulched with 4 inches of straw, the survival may be expected to be slightly better than 50%. These results were obtained on a fairly heavy loam after a winter unusually severe on vegetation, due to the almost complete lack of snow. The Indian species, *A. acuminata*, can obviously not survive without some protection even if it is not cut at all. Mulching has about the same effect on this as on *A. belladonna*.

Additional information was obtained from a number of treatments on sandy soil. In this case mulching with soil and straw and storing of the roots in pits were tried. The percentage of survival resulting from each treatment or procedure was as follows: 85 plants not cut and unprotected, 49.4%; 66 plants cut and unprotected, 33.3%; 105 plants not cut and covered with 3 to 4 inches of soil, 56.2%; 86 plants cut and similarly covered, 31.4%; 105 plants not cut and covered with 2 to 3 inches of straw, 96.1%; 96 plants cut and similarly covered, 77.1%.

Here again in every case the percentage of survival was higher when the plants were not cut back. Covering with soil had no beneficial effect, but a straw mulch was definitely beneficial. These plants were two seasons old, but the survival of those uncut and unprotected was only about half as high as that of the plants of one season's growth on the heavier soil in the replicated plantings.

The roots of some one- and two-year-old plants were dug and placed in pits over winter. Two procedures were used. In one case they were piled up and covered with 6 to 8 inches of soil without a ventilating shaft, and in another they were first covered with 6 inches of straw and then with the soil with provision for some ventilation. In addition some roots were kept in a root cellar. In all cases the stalks were cut close to the ground after the plants were dormant, and the crowns with most of the roots were removed from the ground and stored as stated. The ends of the longer roots usually broke off. In early spring all were planted in plots of 10 plants each, replicated three times. On May 21, a little less than 11% had failed to come up, and examination showed that these were also alive and were slowly developing sprouts. However, the plants were growing very slowly. Only a few were much over 6 inches high when in adjoining rows others left undisturbed, which had survived in the open, were 15 inches high and beginning to bloom.

TABLE VI.—RESULTS OF DRYING MYDRIATIC DRUG PLANTS BY VARIOUS METHODS

Plant	Year	No.	Source, Description, etc.	Date of Harvest	Rapid (Chamber) Drying[a]		Slow Drying		Quality of Product	
					Temperature, ° F.	Time Required,[b] Hr.	Method[c]	Time Required, Days	Alkaloid, %	Color
Belladonna	1941	1	Herb cut about 8 inches above ground	7-24	122–126	29	....	..	0.622	.....
		3	Herb cut about 8 inches above ground	7-28	158–169	18	....	..	0.642	.....
		5	Herb cut about 8 inches above ground	7-29	174–194	5.5	....	..	0.651	.....
		7	Herb cut about 8 inches above ground	7-31	185–194 for 4 hrs., then 126 for 15 hr.	19	....	..	0.660	.....
		1a	Herb cut about 8 inches above ground	7-24	....	..	On wire-bottomed trays	10	0.598	.....
		2	Large leaves removed from stems left by above	7-24	122–126	24	....	..	0.352	.....
	1942	10	Second growth from plants cut close to ground on July 15	9-1	.....	..	On wire-bottomed trays	11	0.554	Brownish green
		9	Second growth from plants cut close to ground on July 15	9-1	.....	..	Tied on sticks	11	0.515	Green
		12	Second growth from plants cut close to ground on July 15	9-1	130–131	20	.....	..	0.503	Bright green
		8	Second growth from plants cut close to ground on July 15	9-2	.....	..	On wire-bottomed trays	10	0.514	Green
		7	Second growth from plants cut close to ground on July 15	9-2	.....	..	Tied on sticks	10	0.538	Green
		11	Second growth from plants cut close to ground on July 15	9-2	165–167	16	....	..	0.515	Bright green
		6	From plants not previously cut	9-1	.....	..	On wire-bottomed trays	9	0.646	Green
		5	From plants not previously cut	9-1	.....	..	Tied on sticks	9	0.656	Green
		4	From plants not previously cut	9-1	144–146	17.5	.....	..	0.671	Bright green
Henbane	1941	9	All the large leaves, except those next to the ground, pulled off at the base	8-6	.....	..	On wire-bottomed trays	9	0.046	.....
		11	All the large leaves, except those next to the ground, pulled off at the base	8-7	120–124	23	....	..	0.064	.....
		1	All the large leaves, except those next to the ground, pulled off at the base	8-1	From 133 to 178 for 5 hr., then reduced to 131 for 16 hr.	21	....	..	0.054	.....
		12	Lower leaves from plants from which No. 11 was obtained	8-7	120–127	23	....	..	0.051	.....
		2	Lower leaves from plants from which No. 1 was obtained	8-1	Same as No. 1	21	.....	..	0.028	.....
		10	Lower leaves from plants from which No. 9 was obtained	8-1	.....	..	On wire-bottomed trays	9	0.029	.....
Stramonium	1941	7	Leaves and tops with stems 3 to 6 inches long[d]	8-12	124–126	26	.....	..	0.574	Grayish brown
		1	Leaves and tops with stems 3 to 6 inches long[d]	8-8	156–160	20	....	..	0.764	Grayish green
		5	Leaves and tops with stems 3 to 6 inches long[d]	8-11	158–187	6	....	..	0.765	Grayish brown

TABLE VI.—RESULTS OF DRYING MYDRIATIC DRUG PLANTS BY VARIOUS METHODS. (*Cont'd. from p. 54.*)

3	Leaves and tops with stems 3 to 6 inches long[4]	8–8	.....	..	On wire-bottomed trays	7	0.760	Grayish green
2	Leaves only	8–8	156–160	20	.....	..	0.474	Brownish green
6	Leaves only	8–11	158–187	6	.....	..	0.539	Greenish brown
4	Leaves only	8–8	.....	..	On wire-bottomed trays	7	0.523	Greenish brown

[a] Electrically heated thermostatically controlled metal chamber with metal trays
[b] Some samples dried in less time than indicated but were kept in the drying chamber until morning; therefore these figures indicate number of hours the drug was exposed to conditions mentioned.
[c] The wire-bottomed trays were placed in a dark attic. The herb tied on sticks was hung up in another attic with more light.
[d] The material for Nos. 7, 1, 5 and 3 of the stramonium, while all of the same nature, was not all picked on the same day or from the same portion of the row. Variation in alkaloid content may be partly due to this fact.

It was observed that many small roots were developing on the date indicated from the ends of broken roots and elsewhere. By the middle of June damage by a stalk-boring insect was so general that these plants could no longer serve to indicate the relative growth rate from roots handled as described.

SUMMARY

Studies were undertaken with mydriatic drug plants to obtain information needed in connection with the domestic production of these plants. Belladonna and henbane were grown in 1942 from seed obtained from various sources to compare yields and quality, and in 1941 general data on yields from small field plots of these and stramonium were obtained. The effects of certain conditions of drying these plants on color and alkaloid content were investigated.

Seed of *Atropa belladonna* from five sources was used. The plants obtained from seed from a sixth source were identified as *A. acuminata*, an Indian species. These were noticeably different from *A. belladonna* and were definitely inferior in productiveness and alkaloid content. The plants from the several seed lots of *A. belladonna* displayed no difference in appearance, but those from one lot were slightly more productive and yielded a drug of somewhathigher alkaloid content. However, the alkaloid content, except that of some of the large stems, was in all cases in excess of the minimum requirement of the U. S. Pharmacopœia.

Hyoscyamus niger was grown from eleven lots of seed obtained from different sources. Three of the lots produced annual plants which were not satisfactory for culture under the prevailing conditions. Considerable difference was found in the biennial types with respect to productiveness and alkaloid content, and all types were damaged by excessive rainfall and poor drainage and by a virus disease. The alkaloid content of all samples of the dried drug was considerably higher than the U. S. Pharmacopœia requirement.

Yields of the several crops were determined from small plantings on light sandy loam in 1941 and on heavier soil in 1942, and the effects of certain harvesting methods on the yields and survival of the plants were observed. Cutting the belladonna plants near the ground was very damaging and reduced the seasonal yield. Cutting 6 to 8 inches above the ground promotes new growth and assures a larger yield. A good second crop of henbane was obtained in 1941, followed by almost complete loss of the plants from a virus disease. In 1942 the plants did not survive long enough to produce a second crop.

The calculated per acre yields of belladonna under various conditions ranged from 299 to 1040 lb., henbane from 196 to 779 lb. and stramonium from 2679 to 2880 lb., dry weight.

A number of methods of drying these drugs were used, ranging from slow drying on screens to drying in a heated chamber at 257° F. The material available for these experiments on drying was too limited to permit repeated tests of the effects of the several conditions mentioned, hence the results cannot be considered conclusive. However, the alkaloid content did not appear to be adversely affected in an important degree by the conditions to which the drug was subjected. Neither of the extreme conditions mentioned gives good green color at all times. Slow drying in trays and by suspending the drug from sticks hung under the roof without artificial heating results in a brown or brownish green color if the drying is prolonged by humid weather. On the whole, the most satisfactory procedure is to subject these drugs to a temperature of 125° F. until dry, but if greater speed is necessary, the temperature may be increased after three or four hours to about 160° F. Complete drying of all three drugs is possible in less

TABLE VII.—EFFECTS OF RAPID DRYING OF BELLADONNA AT HIGH TEMPERATURES

Expt. No.	Procedure	Results—Dryness	Color	Alkaloids, %
38	Hung on sticks under roof.[a] After 5 days when leaves and small stems were dry but large stems tough drying was completed in 3 hr. at 199° F.	Time required: about 5 days	Brownish green	0.569
39	Hung on sticks under roof.[a] After 7 days almost dry. Placed in drier at 200° F. for 1 hr.	Time required: about 7 days	Brownish green	0.532
40	Placed in drier at 4:30 p. m. at 125° F.; out at 8 a. m.	Entirely dry. Time required: 15½ hr.	Green	0.553
41	Placed in drier at 9:00 a. m. at 125° F. At 1:00 p. m. the temperature was raised to 200° F. Out at 6:00 p. m.	Entirely dry. Time required: 9 hr.	Brownish green	0.627
42	Placed in drier at 9:45 a. m. at 122° F. At 10:45 the plants were withered and air-was recirculated 30 min. to raise temperature to 158°, then gradually to 199° by 1:45 p. m. Drug taken out at 4:30 p. m.	Leaves almost dry at 11:45 but stems had dried very little; at 12:45 p. m. the leaves and small stems were brittle. By 1:45 the large stems were leathery but they were not dry until 4:30 p. m. Time required: 7¾ hr.	Green	0.536
43	Placed in drier at 4:40 p. m. at 257° F. and remained at that temperature until 8:00 a. m.	Entirely dry[b]	Dark brown	0.511

[a] Drying takes place more rapidly directly under the roof of a building than in lower rooms.
[b] The drug was no doubt dry early in the night, hence the exposure to this temperature was much longer than necessary.

TABLE VIII.—WINTER SURVIVAL OF BELLADONNA UNDER VARIOUS CONDITIONS

Seed Lot No.	Species	Uncut			Naked			Cut Three Times[a]			Mulched		
		Number of Plants	Alive	Dead	Number of Plants	Alive	Dead	Number of Plants	Alive	Dead	Number of Plants	Alive	Dead
1	*Atropa belladonna*	35	33	2	32	1	31	70	36	34			
2	*Atropa belladonna*	36	36	0	36	0	36	71	24	47			
4	*Atropa belladonna*	36	36	0	35	2	33	72	37	35			
5	*Atropa belladonna*	35	35	0	36	0	36	69	33	36			
6	*Atropa acuminata*	35	5	30	29	0	29	68	31	37			

[a] The plants were cut 6 to 8 inches above the ground on July 15, August 17 and September 29.

than thirty hours under such conditions.

The effects of certain conditions and treatments on winter survival of belladonna plants were noted. Plants one season old, if not cut at all, survived with very small loss; if cut several times, and especially if cut late, very few survived; covering the plants with soil was of very little benefit but a 4-inch covering of straw reduced the loss greatly. Roots taken up and stored in earthen pits with or without straw lived over winter but grew very slowly when replanted in the spring. An Indian species, *A. acuminata*, does not survive without some cover, even if uncut. These observations are based on limited experimental data and the particular seasonal conditions that prevailed.

WHO MAKES IT?

The National Registry of Rare Chemicals, Armour Research Foundation, 33rd, Federal and Dearborn Streets, Chicago, Ill., seeks information on sources of supply for the following chemicals:

Anthraquinone 1-azo-4-dimethylaniline HCl.
2-(*o*-Arsonophenylazo)-*p* cresol
1-(*o*-Arsonophenylazo)-2-naphthol-3,6-disulfonic acid
3-(*o*-Arsonophenylazo)-4,5-dihydroxy-2,7-naphthalene disulfonic acid
Acrylic acid
2-Benzyl pyridine (10 g.)
d-Camphoric anhydride (100 g.)
Cytochrome C
Sym. dialkyl derivatives of succinic acid or its esters (alkyl is methyl, ethyl, propyl, butyl or higher)
l-Lactic acid
Divicine
Ergothionine
Flavone or flavanone
Glucose-1-phosphate
Kynurenine or its sulfate

A New Method for the Assay of Aloin in Aloe*

By Albert C. Smith,† C. B. Jordan and H. G. DeKay

Aloe perryi, known as Socotrine Aloe, and *Aloe vera*, known as Curacao Aloe, were official in the U. S. P. XI (1); while *Aloe ferox*, known as Cape Aloe, was recognized by the U. S. P. X (2). Many species of Aloe have been recognized, deleted and studied for their beneficial effect in medicine.

Aloin was first isolated from Aloe by T. and H. Smith, two pharmacists, in Edinburgh in 1851 (3). Since its isolation many methods have been devised for the removal and assay of Aloin in Aloe. Some of the more prominent methods may be mentioned. They are the methods of Briggs (4), Snyder (5), which is a modification of the Schaffer process (6), Eaton (7), and Viehover (8). Methods for synthesizing Aloin have been attempted by Gardner and co-workers (9).

The chemical nature of Aloin is still uncertain although a number of investigators have attempted to determine its chemical nature. The more prominent workers in this field have been Tschirch (10), Hauser (11), Rosenthaler (12), Leger (13) and Gardner and Joseph (14).

The purpose of this investigation was to study the methods of assay and to develop a suitable procedure for the assay of Aloe for Aloin.

The criteria used as a basis for the development of an assay procedure were: (1) The Aloins obtained should pass the U. S. P. XI solubility test (1); (2) the Aloins should respond to the color tests presented in a second paper; (3) the procedure should be applicable to all species of Aloe studied.

EXPERIMENTAL

The methods of Briggs (4), Snyder (5), and Eaton (7) were studied to determine their suitability as assay procedures.[1]

A study was made on the solubility of Aloin in various immiscible solvents, and from this study solvents were found which could be used for the extraction of Aloin from the drug. Aloin was dissolved in these solvents and a study was made to determine means of precipitating the Aloin by changing the solvent. From this study many different procedures of assay were found possible, but the following was adopted as being the most suitable.

Method of Assay Adopted for Study.—Accurately weigh approximately 15 Gm. of the powdered drug, place in a beaker, add 75 cc. of cold water, 2 cc. of concentrated HCl, and boil for 15 min. Allow the insoluble matter to settle and decant the clear supernatant liquid into a beaker. Add 50 cc. of cold water and 1 cc. of concentrated HCl to the residue, boil for 5 min., allow the resins to settle and decant the clear liquid into the first decanted portion. Add 25 cc. of 20% acidic lead acetate solution (made acidic with acetic acid to litmus paper), filter and wash the lead acetate residue with small portions of distilled water until the washings are colorless. Warm the filtrate, pass H_2S through the warm solution to remove the excess lead, filter and wash the residue with distilled water until the washings are colorless. Evaporate the filtrate to approximately 25 cc. on a steam bath. filter through cotton into a Watkins-Palkins extractor for liquids lighter than water (15), washing the beaker and cotton with cold distilled water until washings are no longer colored. Extract with either *n*-butyl, iso-amyl or secondary amyl alcohol, using a 250-cc. Erlenmeyer flask for the extraction, until the alcoholic layer above the aqueous portion in the extractor is colorless. Remove the extraction flask, reduce the volume of the alcohol to approximately 20 cc. by distillation (do not reduce too low or material will darken) and cool. When the flask is cool, a thick yellow mass appears. Add 200 cc. of petroleum ether, break up the mass with a rubber-tipped stirring rod and allow the precipitate to settle. Filter the clear petroleum ether through a 15-cm. semicrepe filter paper, wash the flask and filter with small portions of petroleum ether and add to filter paper until 250 cc. to 300 cc. have been used. Dry the flask and filter paper in an oven at 60° C. for 1 hr. Allow the flask to cool, then rinse the sides of the flask with approximately 25 cc. of cold distilled water and pour over the filter paper in a beaker. Continue washing the flask and filter with cold water, filtering each washing through a semicrepe filter into a clean beaker until the washings are no longer colored. (Do not use hot water as some of the resinous material is soluble in hot water. Nearly all the resinous material can be removed in this step.) Evaporate the filtrate to approximately 25 cc., filter through cotton into a Watkins-Palkins extractor for liquids lighter than water, washing the beaker and cotton with small portions of cold water until washings are colorless. Extract with either *n*-butyl, iso-amyl or secondary amyl alcohol until the alcoholic layer above the aqueous liquid in the extractor is colorless. Remove the extraction flask, reduce the volume of alcohol to approximately 25 cc. as before, cool, add 200 cc. of petroleum ether, stir to break up the residue, allow the precipitate to settle and filter the clear petroleum ether solution through a tared filter paper, add 100 cc. of petroleum ether to the flask and transfer the precipitate to the tared filter paper. Remove the adhering pieces of Aloin from the flask with a rubber-tipped stirring rod and wash into the tared filter paper. Continue washing the flask and residue with several portions of petroleum ether to remove the alcohol. Dry in a desiccator for one hour, and then in an oven at 60° C. for 12 hr., cool and weigh.

It will be noted in particular that the yield of Aloin from Cape Aloe by the New Method far exceeds any assay or procedure submitted up to the

---

* The work was begun under the late Dean C. B. Jordan and completed under H. Geo. DeKay, Professor of Pharmacy, Purdue University, Lafayette, Ind.

† An abstract of a thesis presented as partial fulfillment for the degree of Doctor of Philosophy, Purdue University, Lafayette, Ind., May, 1942.

[1] The complete data and results from these methods will be found in the original thesis.

TABLE I.—THE RESULTS OBTAINED FROM THE ASSAYS OF THE THREE ALOES BY (1) THE NEW METHOD, (2) THE SYNDER METHOD AND (3) THE EATON METHOD

Curacao Aloe, % of Aloin		Socotrine Aloe, % of Aloin		Cape Aloe, % of Aloin	
(1) The New Method					
14.75[a]	11.79[a]	4.41[a]	5.83[a]	20.64[b]	31.22[a]
...	11.83[d]	5.12[d]	10.90[f]	21.81[b]	22.61[b]
11.88[a]	12.61[a]	9.99[f]	12.36[a]	23.47[b]	25.39[a]
18.90[a]	18.29[a]	10.56[f]	9.31[f]	23.43[b]	23.42[b]
17.00[a]	15.97[a]	11.25[a]	9.86[f]	23.25[b]	19.05[b]
17.47[a]	12.29[a]	10.99[f]	11.26[f]	20.57[b]	21.78[b]
11.99[a]	13.73[a]	6.97[a]	12.29[f]	22.69[b]	22.23[b]
14.26[a]	15.28[a]	10.56[f]	12.00[f]	17.23[b]	21.70[b]
14.80[a]	18.27[a]	9.52[f]	12.05[f]	...	22.12[b]
(2) The Snyder Method					
13.91	15.62	16.52[h]	7.31	8.40	7.88
14.24	...	10.04	...	11.55[h]	...
(3) The Eaton Method					
36.40[i]	28.64[i]	17.79[i]	12.99[i]	15.24[i]	14.71[i]
27.56[i]		13.15[i]		15.96[i]	
19.60[h]	24.93[h]	15.11[h]	13.22[h]	13.34[h]	16.25[h]

[a] The residues were a very light yellow color.
[b] The residues were a pale yellow color and very fluffy.
[c] The residues were a dark yellow color.
[d] The residues were distilled too low and were slightly burned.
[e] The residues were a tan color.
[f] The residues were the same color as commercial Aloin.
[g] The residues were a lemon-yellow color.
[h] The products formed in these samples were a very dark brown color.
[i] The confirmatory acetylation tests were made on these samples (Eaton).
[k] The U. S. P. XI solubility tests were made on these samples.

present time. The assays for the Curacao approach concordance; although not ideal, they do give a greater degree of accuracy than some methods which have been submitted.

TABLE II.—THE RESULTS OBTAINED BY DETERMINING THE U. S. P. XI SOLUBILITY TESTS ON THE RESIDUES OF (1) THE NEW METHOD, (2) THE EATON METHOD

Curacao Aloe, % of Aloin		Socotrine Aloe, % of Aloin		Cape Aloe, % of Aloin	
(1) The New Method					
0.30	0.25	0.75	0.67	0.30	0.34
(2) The Eaton Method					
40.84	..	42.33	..	24.71	..

TABLE III.—THE RESULTS OBTAINED BY THE CONFIRMATORY ACETYLATION TEST DESCRIBED BY EATON

Curacao Aloe, % of Aloin		Socotrine Aloe, % of Aloin		Cape Aloe, % of Aloin	
33.81	9.35	18.56	9.07	11.19	17.26
27.56	..	13.15	..	15.96	...

All samples responded to the color tests. The products obtained by the Snyder method of assay were darker in color than the ones obtained by the new process. In the Eaton method, the final products before acetylation appeared resinous and had a yellowish orange color. The flasks on drying had the appearance of being shellacked.

SUMMARY AND CONCLUSIONS

1. The process described is equally well adapted for all species of Aloe studied. The final product passes the U. S. P. XI solubility test for Aloin.

2. The Snyder method of assay was found to be best adapted for Barbadoes and Curacao Aloe.

3. The E. O. Eaton method gave high results. The extractive did not pass the solubility test.

4. Cape Aloe, which was deleted from the U. S. P. XI, appears to contain the highest percentage of Aloin.

5. It is recommended that further study of the chemical nature of Aloin be made in an effort to find a simpler method of assay.

REFERENCES

(1) "United States Pharmacopœia," Eleventh revision, Mack Printing Company, Easton, Pa., 1936, p. 47.
(2) "United States Pharmacopœia," Tenth revision, Mack Printing Company, Easton, Pa., 1926, p. 41.
(3) Smith, T. and H., *Pharm. J.*, 11 (1852), 30.
(4) Briggs, C. H., THIS JOURNAL, 12 (1923), 774.
(5) Snyder, J. P., *U. S. P. Bull.*, Subcommittee No. 6, third page following page 76.
(6) Schaffer, G. L., *Pharm. J.*, 58 (1897), 287.
(7) Eaton, E. O., *J. Assoc. Official Agr. Chem.*, 14 (1931), 311.
(8) Viehover, A., *Am. J. Pharm.*, 107 (1935), 47.
(9) Foster, H., and Gardner, J. H., *J. Am. Chem. Soc.*, 58 (1936), 597.
(10) Tschirch, A., "Handbuch der Pharmakognosie," Band I, Abteilung 2, Chr. Herm Tauchnitz, Leipzig, 1910.
(11) Hauser, F., *Pharm. Acta Helv.*, 6 (1931), 79.
(12) Rosenthaler, L., *Arch. Pharm.*, 270 (1932), 214.
(13) Leger, E., *Boll. soc. chim.*, (5), 3 (1936), 435, through *C. A.*, 30 (1936), 383.
(14) Gardner, J. H., and Joseph L., THIS JOURNAL, 25 (1937), 794.
(15) Palkins, S., Murray, A. G., and Watkins, H. R., *J. Ind. Eng. Chem.*, 17 (1925), 612.

Color Tests for Aloe and Aloin*

By Albert C. Smith, C. B. Jordan† and H. G. DeKay‡*

In the study of "A New Method for the Assay of Aloin in Aloe" many reagents were used to determine the suitability of various chemicals which might possibly be used as a colorimetric assay for Aloe. Many new color tests were made as well as those recognized by the U. S. P. XI (1) and other Pharmacopœias under tests for identity of the drug and Aloin.

Many color tests are outlined in the literature for the identification of the drug and its active agent. Some of the more prominent tests may be mentioned. They are the Borntrager test (2), the Klunge Cupra-Aloin test (3), the Cripps-Dymond test (4), the Schoutelen test (5) and the Heuberger Cyanogen reaction (6).

EXPERIMENTAL

Solutions of the drug and Aloins were made in various immiscible solvents and water. The clear supernatant liquid was decanted from the undissolved matter. To 5-cc. quantities of these solutions a large number of alkaloidal precipitants, U. S. P. XI test solutions and qualitative reagents were added. The following data and experimental work are in outline form describing the tests that show the greatest promise in the identification of the various species of Aloe and Aloin studied.

I. QUALITATIVE TESTS ON THE ALOINS

A. Make a saturated aqueous solution of the Aloins. To 5 cc. of this solution in separate test tubes add:

1. One cc. of 50% sodium hydroxide solution.
2. Three cc. of concentrated nitric acid.
3. Two cc. of phosphomolybdic acid T. S. and 3 cc. of stronger ammonia water.
4. One cc. of 5% ferric chloride solution.
5. One cc. of saturated solution of mercuric chloride.

The color reactions are indicated in Table I.

B. Dissolve 0.5 Gm. of the Aloin in 25 cc. of anhydrous acetone. To 5 cc. of this solution in separate test tubes add:

1. Three cc. of bromine water.
2. One cc. of 1% vanadium oxychloride in carbon tetrachloride.

The color residues are indicated in Table II.

TABLE I

	Aloin from Curacao Aloe	Aloin from Socotrine Aloe	Aloin from Cape Aloe	Aloin from Barbadoes Aloe	Aloin from Commercial Aloe
1.	Red colored solution	Deep red color. Yellow fluorescence	Reddish yellow fading to yellow	Dark red solution	Deep red solution
2.	Brilliant pink changing to deep red	Yellowish red fading to yellow-orange	Yellow color changing to green	Brilliant red color	Brilliant pink color
3.	Greenish black solution changing to black	Deep, green cloudy solution slowly darkens	Bluish green, cloudy solution changing to olive-green	Brownish black cloudy solution changing to black	Green color, slowly changing to brown and fading
4.	Black solution	Black solution	Dark brown solution changing to black	Cloudy black solution with precipitation	Rapidly turns black
5.	No change in color	Pink fluorescence	No change in color	No change in color	Turns lighter in color. Pink fluorescence

TABLE II

	Aloin from Curacao Aloe	Aloin from Socotrine Aloe	Aloin from Cape Aloe	Aloin from Barbadoes Aloe	Aloin from Commercial Aloe
1.	Brilliant pink changing to red	No change in color	Pink color with fluorescence	Brilliant pink color changing to deep red	Pink solution changing to a brilliant red
2.	Deep, green color with muddy appearance	Deep green with muddy appearance	Deep, cloudy green color	Brownish green color changing to a deeper tone	Green-colored solution

II. QUALITATIVE TESTS ON THE DRUG

A. Add 100 cc. of distilled water to 5 Gm. of the drug and heat to boiling, allow to cool and decant the clear liquid. To 5 cc. of this decanted liquid in separate test tubes add 3 cc. of bromine water.

The color reactions and nature of the precipitate are given in Table III.

---

* An abstract of a thesis presented as partial fulfillment for the degree of Doctor of Philosophy, Purdue University, Lafayette, Ind., May, 1942.

† This work was initiated and carried on under the supervision of the late Dean C. B. Jordan.

‡ Professor of Pharmacy, Purdue University, Lafayette, Ind.

B. Add 25 cc. of anhydrous acetone to 1 Gm. of the drug, heat to boiling, allow to cool and decant the clear liquid. To 5 cc. of this solution in separate test tubes add:

1. One cc. of 1% vanadium oxychloride in carbon tetrachloride.
2. Three cc. of bromine water.

The observations are recorded in Table IV.

SUMMARY AND CONCLUSIONS

1. Color tests when applied to Aloins obtained from different species of the drug do not give the same results.

It is suggested that a wide variety of color tests be established for identification purposes.

TABLE III

Curacao Aloe	Socotrine Aloe	Cape Aloe	Barbadoes Aloe	Exhausted Aloe
Wine-red or purplish red solution. Compact bluish black precipitate	Deep, orange-yellow solution with a stringy, pale yellow precipitate	Deep, orange-yellow solution with flocculent, yellow precipitate	Deep, red solution with a dark flocculent precipitate	Deep, yellow solution with a flocculent orange-yellow precipitate

TABLE IV

	Curacao Aloe	Socotrine Aloe	Cape Aloe	Barbadoes Aloe	Exhausted Aloe
1.	Deep, brownish green precipitate	Deep, green colored solution	Light, gray-green precipitate. Brown colored solution	Flocculent, light grayish green precipitate. Reddish brown solution	Flocculent, light grayish green precipitate. Reddish brown solution
2.	Brilliant pink color. Pink to yellow fluorescence	No change in color	Yellow solution with pink fluorescence	Dirty, red color changing to brick-red	Deep, brownish red solution. Turns darker

REFERENCES

(1) "United States Pharmacopœia," Eleventh revision, Mack Printing Co., Easton, Pa., 1936, p. 47.
(2) Borntrager, H., *Z. anal. Chem.*, 19 (1880), 165; through *Yearbook Pharm.* (1880), 140.
(3) Klunge, A., *Schweiz. Wochenschr. Pharm.*, 18 (1882), 170; through *Arch. Pharm.*, 221 (1883), 363.
(4) Cripps, R. A., and Dymond, T. S., *Pharm. J.* 44 (1885), 632.
(5) Schoutelen, L., *Z. allgem. österr. Apoth.-Ver.*, 46 (1892), 249.
(6) Heuberger, K., *Schweiz. Wochs. Chem. Pharm.* (1899), 56.

WHO MAKES IT?

The National Registry of Rare Chemicals, Armour Research Foundation, 33rd, Federal and Dearborn Streets, Chicago, Ill., seeks information on sources of supply for the following chemicals:

7,8-Dihydroxy-4-methyl coumarine (methyl daphnetin)
β-Hydroxy-glutamic acid-*d*
Mellitic acid
Orotic acid
Potassium salt of tetrabromphenolphthalein ethyl ester
Adenylic acid (muscle)
Morin, pure
o-Phthalic aldehyde
Sodium acid citrate
Cytosine
Chrysophanic acid, 25 grams
Cascara emodin, 25 grams
Dihydrotachysterol
Hydroquinone monobenzyl ether
Lumisterol
Methyl violegen
Dehydropropiorhodanine
Suprasterol
Tachysterol
Toxisterol
Tetrahydro isoquinoline hydrochloride
Xanthopterin
4-Methyl, 6-methoxy quinoline
Rubrine
Decamethylene diguanidine
N-ethyl, 8-hydroxy tetrahydroquinoline
Tumenol

Influence of Age of Rabbits on the Toxicity of Ouabain*,†

By K. K. Chen and E. Brown Robbins‡

The influence of age on the relative susceptibility to digitalis of young and old subjects has been investigated from time to time. That it is not a settled problem is evidenced by the résumé of Haag and Corbell (1). One of the principal difficulties in accurate, experimental investigations has been the scarcity of animals of known ages; for example, cats. In this laboratory, a strain of albino rabbits became available for study which had originally been raised for show purposes by an outside breeder. Their birth dates were individually recorded, and some of the animals reached a known age of 5 to 6 yr. in apparently good health. Thus, an opportunity was afforded to test the effect of age on sensitivity to ouabain. The problem of the effect of age on drug action was suggested to us by Dr. Edward J. Stieglitz, Consultant in Gerontology, National Institute of Health. His untiring advice and encouragement in this work are hereby acknowledged.

The experimental procedure was the same as that for cats (2). Briefly, after the rabbit was anesthetized with ether, ouabain in a 1:100,000 solution was injected intravenously from a burette at the rate of 1 cc. per minute. In rabbits about 6 wk. of age the jugular vein was used, but in all older animals the femoral vein was cannulated. The end point was determined by means of a stethoscope—the injection being terminated as soon as the heart sound disappeared. The heart of each animal was carefully dissected and weighed. The lethal dose was calculated by both the body weight and the heart weight. The mean lethal dose is the antilogarithm of the mean of logarithms of individual doses.

TABLE I.—TOXICITY OF OUABAIN BY INTRAVENOUS INJECTION IN RABBITS OF DIFFERENT AGE GROUPS

Rabbit Number	Sex	Age Group Mo.	Age Group Days	Body Weight, Kg.	Heart Weight, Gm.	Lethal Dose by Body Weight, μg/Kg.	Lethal Dose by Heart Weight, μg/Gm.	Mean Lethal Dose ± S. E. by Body Weight, μg/Kg.	Mean Lethal Dose ± S. E. by Heart Weight, μg/Gm.
1	M		40	0.978	2.8	163.6	57.1		
2	M		40	0.826	2.1	257.9	101.4		
3	M		40	0.780	2.3	155.8	52.8		
4	F		42	1.039	2.8	221.4	82.1	197.8 ± 13.5	70.2 ± 6.1
5	M		42	1.009	2.8	222.5	80.2		
6	F		42	0.970	2.7	184.0	66.1		
7	F		44	0.944	3.0	199.2	62.7		
8	F	3	4	2.602	6.2	176.4	74.0		
9	F	3	4	2.630	5.7	154.4	71.2		
10	M	3	4	2.533	7.0	98.3	35.6		
11	M	3	4	2.816	7.1	90.6	35.9	120.3 ± 10.6	52.0 ± 5.3
12	F	3	11	3.523	7.1	104.3	51.8		
13	F	3	11	3.151	7.1	151.9	67.4		
14	F	3	11	2.715	5.9	105.3	48.5		
15	F	3	11	3.187	7.4	108.6	46.8		
16	M	6	3	3.770	9.35	127.2	51.3		
17	M	6	3	3.557	7.6	88.8	41.6	110.3 ± 12.1	49.0 ± 4.1
18	M	6	3	3.157	6.8	118.6	55.1		
19	F	11	19	3.218	6.3	102.1	52.1		
20	F	11	19	3.813	8.0	129.6	61.8	117.8 ± 11.1	55.8 ± 4.3
21	M	12	4	4.062	9.2	146.5	64.7		
22	M	12	4	3.834	8.2	99.4	46.5		
23	M	24		4.182	9.4	137.7	61.3		
24	M	24		4.773	9.4	73.7	37.4	116.6 ± 20.9	55.2 ± 9.8
25	M	25		3.842	7.6	169.7	85.8		
26	M	25		3.205	7.3	107.3	47.1		
27	M	37		3.881	8.0	126.5	61.4		
28	F	37		4.924	11.0	69.5	31.1		
29	F	39		4.982	10.3	97.7	47.2	82.9 ± 13.3	40.7 ± 7.3
30	F	39		5.208	11.0	49.3	23.4		
31	F	62		5.658	9.9	92.4	52.8		

* Received July 28, 1943, from the Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, Ind.

† The authors are indebted to Messrs. Robert C. Anderson and John C. Hanson for their invaluable assistance in the experiments.

‡ Now Lieutenant, U. S. N. R.

The results of our experiments are summarized in Table I. It is apparent that rabbits are least susceptible to ouabain in the age group of 40–44 days. Those aged 3–25 mo. have a relatively higher susceptibility with very little difference in the 4 groups studied—aged approximately 3 and 6 mo., and 1 and 2 yr. There is a further increase in susceptibility when rabbits reach the age of 37–62 mo. (about 3–5 yr.).

It has been repeatedly suggested (3, 4) that the lethal dose based on heart weight is more accurate than that based on body weight since digitalis and its allies act principally on the heart. In the age groups (approximate) of our rabbits, the average heart weights in percentage of body weights were as follows: 40–44 days, 0.282%; 3 mo., 0.232%; 6 mo., 0.225%; 1 yr., 0.195%; 2 yr., 0.211%; and 3–5 yr., 0.203%. The heart weight of the youngest group is relatively larger than that of other groups. However, it does not nullify the differences in results, for rabbits aged 40–44 days still prove to be less susceptible than those in the older age groups. Regarding rabbits aged 3–5 yr., the mean lethal dose is significantly smaller than that of rabbits aged $^1/_4$–2 yr. if calculated by the body weight. If based on the heart weight, however, the difference is not significant.

SUMMARY

1. Evidence has been presented to show the influence of age of rabbits on their susceptibility to ouabain when injected intravenously.

2. If the mean lethal doses based on body weight are compared, rabbits aged 40–44 days are the least susceptible; those aged 3–25 mo., next in order; and those aged approximately 3–5 yr., the most susceptible.

3. Similarly, if the mean lethal doses based on heart weight are compared, rabbits aged 40–44 days are significantly less susceptible to ouabain than older rabbits.

REFERENCES

(1) Haag, H. B., and Corbell, R. L., Jr., *J. Pharmacol.*, 68 (1940), 45.

(2) Chen, K. K., Jensen, H., and Chen, A. L., *Ibid.*, 43 (1931), 13.

(3) Bond, W. R., THIS JOURNAL, 16 (1927), 137.

(4) Chen, K. K., Bliss, C. I., and Robbins, E. B., *J. Pharmacol.*, 74 (1942), 223.

Influence of Age of Mice on the Toxicity of Alcohol*

By K. K. Chen and E. Brown Robbins†

In continuation of our studies on the effect of age on the action of drugs, ethanol was injected subcutaneously into mice. This animal has a life span of 2 to 3 yrs. It is said that a mouse 2 yrs. of age is comparable to a person 80 yrs. old. The purpose of the present investigation was to determine the toxicity of alcohol by a single dose in mice of different ages—both male and female. They were inbred animals of the same strain. Absolute ethanol was diluted with water to a 20% solution by weight. The temperature of the room in which the experiments were carried out varied from 26° to 28° C.

The results are shown in Table I, and the median lethal doses (LD_{50} ± standard error), computed by their common slopes (1), are summarized in Table II. Except in the oldest group of female mice, 24 mos. of age, there is a strong indication that the younger the animal, the lower the susceptibility to alcohol. Since the data for the male and the female animals resemble each other, they are combined, and the LD_{50}'s recomputed, as shown in the last column of Table II. If these figures are compared, the differences between the LD_{50}'s of mice 4 and 6 mos. old, and 12 and 24 mos. old, respectively, are significant. The difference between animals 1 and 4 mos. old is not significant. Weaning at the end of 1 mo. of age apparently has no effect on the animal's response. The apparent decrease in susceptibility at 24 mos. of age is obviously due to the unexpected but uniform response of the females.

The results enumerated above correspond, in general, to the findings of Pearl, White and Miner (2). These authors demonstrated that the older the fruit fly, *Drosophila melanogaster*, the shorter the time of

* Received July 28, 1943, from the Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, Ind.

† Now Lieutenant, U. S. N. R.

induction of anesthesia by a standard dose of absolute alcohol. Apparently the high metabolic rate of very young animals increases the oxidation of alcohol in tissues. To repeat, the present experiments were carried out for the sole purpose of determining the toxicity of alcohol in different age groups of mice by a single dose. The results do not necessarily contribute information to the question of repeated or continuous imbibition of alcohol by man. Certainly it must not be construed that young human subjects can tolerate alcohol better than adults.

TABLE I.—TOXICITY OF ETHANOL IN DIFFERENT AGE GROUPS OF MICE (SUBCUTANEOUS INJECTION)

Age, Mo.	Dose, Gm./Kg.	Number of Mice Died/Number Used	
		Males	Females
1	6.0	1/10	...
	7.0	3/10	0/8
	8.0	2/10	1/8
	9.6	5/10	4/8
	11.0	8/11	7/8
4	6.0	1/10	1/10
	7.0	0/10	0/10
	8.0	4/10	1/10
	9.6	9/10	3/10
	11.0	13/13	9/9
6	6.0	0/7	2/10
	7.0	3/8	1/10
	8.0	6/8	5/10
	9.6	8/8	10/10
	11.0	...	12/12
12	4.2	1/6	0/7
	5.0	0/10	0/8
	6.0	2/10	3/10
	7.0	2/10	3/10
	8.0	2/8	7/10
	9.6	8/8	7/8
	11.0	6/6	...
24	6.0	1/10	0/4
	7.2	4/10	1/8
	8.4	7/10	2/8
	10.0	...	3/8
	12.0	...	5/5

TABLE II.—MEDIAN LETHAL DOSES OF ETHANOL IN MICE OF DIFFERENT AGE GROUPS

Age, Mo.	LD_{50} ± S. E.: Gm./Kg.		
	Males	Females	Combined
1	9.035 ± 0.431	9.570 ± 0.469	9.255 ± 0.332
4	8.168 ± 0.359	9.436 ± 0.418	8.840 ± 0.282
6	7.318 ± 0.400	7.663 ± 0.317	7.563 ± 0.258
12	7.676 ± 0.363	7.298 ± 0.302	7.491 ± 0.247
24	7.639 ± 0.440	9.687 ± 0.531	8.612 ± 0.377

SUMMARY

The toxicity of ethanol has been determined by subcutaneous injection in different age groups of mice—from 1 to 24 mo. The results appear to indicate that the older the mouse the higher the susceptibility.

For some unknown reason, the female mice 24 mo. of age failed to show this tendency. In fact, their response was more like that of the mice 1 mo. of age.

REFERENCES

(1) Miller, L. C., Bliss, C. I., and Braun, H. A., THIS JOURNAL, 28 (1939), 644.

(2) Pearl, R., White, F. B., and Miner, J. R., *Proc. Natl. Acad. Sci.*, 15 (1929), 425.

Book Reviews

The Optical Properties of Organic Compounds, by ALEXANDER N. WINCHELL, The University of Wisconsin. The University of Wisconsin Press, Madison, Wis., 1943. xiii + 342 pp., 17 x 26 cm. Planographed. Price, cloth, $5.00.

This publication is somewhat similar in its general arrangement to the author's earlier work on artificial inorganic solid substances, not containing, however, as much introductory descriptive material on crystallography and the immersion method as the former publication. As the author has stated in the introduction, "the aim has been to include all organic compounds whose optical properties are sufficiently well known to permit identification by optical methods." The arrangement of the compounds is based on the fourth edition of Beilstein's "Handbuch der organischen Chemie." The large groups are the acyclic, isocyclic and heterocyclic compounds, respectively. A fourth grouping includes the carotenoids, carbohydrates, steroids, alkaloids, and nitrogen-free substances. References apparently are given for all the data, and there is included a determinative table based on refringence. Some one-thousand entries are listed in the index. In addition to the optical crystallographic data given for the compounds, there are also many figures illustrating crystal habit. The choice of chemical names for many of the substances is admitted to be arbitrary, but these are frequently accompanied by common names well known to the chemist.

The French system of refractive index terminology is used, and the index values frequently stated to the fourth decimal place. Some workers are more accustomed in this country to the German system for the refractive index symbols where the Greek letter subscripts are used with the conventional sign (n) for the refractive index.

The optical data here presented again impress the reviewer with their importance as characterizing crystalline substances. Quite frequently other physical constants, particularly the melting points, are not so significant as desired. In fact, many instances can be recalled by the writer where a sharp melting point could not be obtained on an organic crystalline substance, decomposition ensuing before there was any evidence of softening. By a determination of the refractive indices and other optical crystallographic data, additional diagnostic constants are available, these being readily determined and at the expenditure of a very small amount of time and material. Eventually such data will be included in any description of a crystalline substance if that description is to be considered complete. The book here briefly reviewed furnishes considerable data for a beginning of that sort.

It is to be hoped that a new edition of this work will be more substantially bound and produced on a higher quality of paper. Such a book will be referred to constantly and there is some question as to whether it will stand hard usage in its present form.—GEORGE L. KEENAN.

The Theory of Emulsions and Their Technical Treatment, by WILLIAM CLAYTON. Fourth Edition. The Blakiston Company, Philadelphia, 1943. vi + 492 pp., 103 illus., 15 x 24.7 cm. Price, $10.00.

Since the printing of the first edition in 1923, there has been no substitute for this excellent handbook of Clayton. The careful balance between the theoretical and the practical, the judicious choice of material from the literature and the manner of presentation have all been combined to create a book worthy of a prominent place on the desk of all interested in the technology of emulsions.

This new edition is characterized by the deletion of certain older material and the introduction of modern findings. To introduce the newer concepts in their proper setting, the first few chapters have been rearranged. The other chapters follow the traditional pattern of former editions in bold outline, but much new material has been added.

To the colloidal chemist, the material on wetting agents, modern concepts of surface chemistry and certain present-day trends in physical chemistry will be of particular interest. Pharmaceutical chemists will be especially grateful for the interpretation of phase inversions.

Emulsions play such an important role in pharmaceutical chemistry that no laboratory can afford to be without this book. The reviewer can think of no volume more worthy of use as a text for graduate students in pharmacy schools, since colloidal phenomena relating to emulsions are clearly and concisely presented in a teachable manner. Without over-simplification, these phenomena are described with a minimum of mathematical terminology.

The quality of workmanship in this handbook leaves nothing to be desired.—M. W. GREEN.

Introduction to Organic and Biological Chemistry, by L. EARLE ARNOW, Sharpe and Dohme, Inc., and HENRY C. REITZ, Western Regional Research Laboratory. The C. V. Mosby Company, St. Louis, Mo., 1943. 736 pp., 13.5 x 21.5 cm. Price, $4.25.

This book is unique in that it combines, in one volume of 736 pages, discussions of both elementary organic and biological chemistry. In writing the text the authors "have endeavored to include material that will be both useful and interesting to students in premedical, predental, home economics, agriculture, dietetics, and physical education courses, as well as to others who may be interested in the correlation of chemistry with biological sciences."

The text is arranged in three parts. Part I is a review of chemical fundamentals. Fifty-three pages are devoted to a recapitulation of "Chemical Facts and Theories" and a discussion of the behavior of "Particles in Solution." The authors do not anticipate that the instructor shall use class time to cover this material but that it may be assigned for reading. Part II, 482 pages, is devoted to a discussion of elementary organic chemistry. The scheme of development is similar to that used in most organic chemistry texts, 318 pages being alloted to the aliphatic compounds and the remaining 164 pages to the aromatic compounds. Many references are made to the applications of the reactions and products discussed. These are further emphasized by the inclusion of many interesting illustrations. "Every effort has been made to relate, in so far as possible, the study of organic chemistry to the everyday life of the student."

Part III, 159 pages, covers the following topics: Carbohydrate Metabolism, Lipid Metabolism, Protein Metabolism, Inorganic Metabolism, Hormones, Vitamins, and Summary of Nutritional Requirements. As these titles indicate, this part is a "summary of the more important phases of metabolism and nutrition." In preparing this section the authors "have followed a chemical, rather than the more usual physiological or anatomical outline." They believe that considerable space has been saved without the omission of essential material since "special chapters on digestion, absorption, blood, and excretion were made unnecessary."

The Appendix of 17 pages is of exceptional interest because of the inclusion of seven tables on the "Composition and Caloric Value of Foods." Column headings are as follows: percentage by weight of Protein, Carbohydrate, Fat, Crude Fiber, Ash, Calcium, and Phosphorus, milligrams per kilogram of Iron and Copper, Alkaline or Acid Effect (cc. of $N/1$ reagent per 100 Gm. or 100 cc. of food), calories per 100 Gm., Vitamin Content, and Size, Weight, and Total Calories in an average portion of the food.

Other features which merit mention are (*a*) suggestions of techniques and methods for the successful study of organic chemistry at the beginning of Part II and (*b*) the selected references included at the end of most chapters. These have been chosen for their extensive bibliographies as well as their pertinent information.

The book should serve adequately as a textbook for courses of the type mentioned above and as a reference book for the pharmacist and pharmaceutical chemist.—E. L. CATALINE.